THE UNITED STATES PATENT AND TRADEMARK OFFICE
BOARD OF PATENT APPEALS AND INTERFERENCES

In Re Application of: James P. Elia)	
)	Group Art Unit: 1647
Serial No.: 09/064,000)	
)	Examiner: Daniel C. Gamett
Filed: April 21, 1998)	
)	
For: METHOD FOR GROWTH)	Client Docket No. Case No. 1
OF SOFT TISSUE)	

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APPEAL BRIEF

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Dated: <u>August 17, 2009</u>

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REAL PARTY IN INTEREST

The real parties in interest in the instant appeal are Assignees, Dental Marketing Specialists, Inc., an Arizona corporation, 9377 E. Bell Road, Suite 385 Scottsdale, Arizona 85260, and Jerry W. Bains and Salee C. Bains Irrevocable Trust, 9013 Red Lawrence Drive, Carefree, Arizona 85377. Subsequent to the assignment recordal for the instant application, the address of Dental Marketing Specialists, Inc. changed to 7364 East Crimson Sky Trail, Scottsdale, Arizona 85262. Also, subsequent to the assignment recordal for the instant application, the address of Jerry W. Bains and Salee C. Bains Irrevocable Trust changed to 39096 N. 102nd Way, Scottsdale, Arizona 85262.

RELATED APPEALS AND INTERFERENCES

There are no related appeals, interferences, or judicial proceedings known to Appellant, Appellants' legal representative, or Assignee, which may be related to, directly affect, be directly affected by, or may have a bearing on the Board's decision in the pending appeal, except for the following:

1.   Co-pending application Serial No. 09/794,456, in which Appellant's Appeal Brief was filed at the Patent and Trademark Office (hereinafter "the PTO") on February 6, 2009;

2.   Co-pending application Serial No. 09/836,750, in which Appellant's Appeal Brief was filed at the PTO on May 27, 2009; and

3.   Co-pending application Serial No. 10/179,589, in which Appellant's Appeal Brief was filed at the PTO on July 2, 2009.

The attached Related Appeals and Interferences Appendix confirms such statement.

STATUS OF CLAIMS AND CLAIMS UNDER APPEAL

Claims 1-6 were cancelled in the Amendment filed February 15, 2001.

Claims 7-191 were cancelled in the Amendment filed September 3, 2002.

Claims 192-381 were cancelled in the Amendment filed August 19, 2004.

Claims 382-402 and 406 were cancelled in the Amendment filed November 28, 2007.

In view of the above-identified cancellation of claims 1-402 and 406, the correctness of the February 26, 2009, Non-Final Rejection (hereinafter "the Rejection") made by the PTO of claims 403-405 and 407-412 is being appealed. Specifically, the instant appeal includes the rejection of claims 403-405 and 407-412 under 35 U.S.C. §112, second paragraph, as being indefinite; the rejection of claim 404 under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement; the rejection of claims 403-405 and 407-412 under 35 U.S.C. §112, first paragraph, as failing to comply with the enablement requirement; the provisional rejection of claims 403-405 on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 163 and 170-173 of co-pending application Serial No. 10/179,589; and the new provisional rejection of claims 382-406 on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 161-164 and 172-174 of co-pending application Serial No. 10/179,589.

STATUS OF AMENDMENTS

No amendment has been made or entered subsequent to the Rejection.

SUMMARY OF CLAIMED SUBJECT MATTER

Appellant's invention is directed to a method of growing and integrating a desired artery at a selected site in the body of a human patient. Claim 403, the only independent claim on appeal, recites a method comprising locally injecting stem cells in the body at a selected site, forming a bud (primordium) at said site, and growing an artery from said bud. This method is described in the instant specification at page 20, lines 10 thru page 21, line 15; page 30, line 14 thru page 32, line 19; page 33, lines 8–10; page 37, lines 19-25; pages 40–42 and 44–48 and 52. Claim 404 depends from claim 403 by requiring growth of an artery in the leg of a patient by intramuscular injection of stem cells. Intramuscular injection to grow an artery in a patient's leg is described on pages 45, and in Example 18, page 54. Claim 407 depends from claim 403 and defines the stem cell as being harvested from bone marrow; claim 408 depends from claim 407 and defines obtaining bone marrow from the patient; claim 409 depends from 403 and requires using bone marrow obtained from peripheral blood; and claim 410 depends from claim 409 and requires using the patient's blood. The specification describes the subject matter of claims 407-410 at pages 40–42 and pages 47–52, wherein the specification discloses using a patient's own stem cells for growing multiple described organ species, through differentiation and morphogenesis. The organ species artery is specifically disclosed as a desired target organ on page 52. Additionally, pluripotent stem cells are described at page 50. Claims 411 and 412 define determining blood flow through the artery and observing the grown artery. The specification discloses such procedures in Examples 18 and 19, pages 54 and 56.

GROUNDS OF REJECTION FOR REVIEW ON APPEAL

Claims 403-405 and 407-412 were rejected under 35 U.S.C. §112, second paragraph, as being indefinite.

Claim 404 was rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement.

Claims 403-405 and 407-412 were rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the enablement requirement.

Claims 403-405 were provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 163 and 170-173 of co-pending application Serial No. 10/179,589.

Claims 382-406 were newly provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 161-164 and 172-174 of co-pending application Serial No. 10/179,589.

ARGUMENT

Rejection of Claims 403-405 and 407-412
under 35 U.S.C. §112, Second Paragraph – Indefiniteness

Claims 403-405 and 407-412 stand rejected as indefinite under 35 USC 112, second paragraph for failing to particularly point out and distinctly claim the invention. Specifically, the PTO contends that step (b) in calling for "forming a bud" is indefinite since it not clear what action is required by the practitioner in performing the recited method. Appellant disagrees that the subject matter of claims 403-405 and 407-412 lacks the definiteness required by Section 112 of the Statute.

The Rejection incorrectly states that "the specification does not provide any teaching specifically directed to forming a bud." The specification at page 31, lines 18-26 discloses that once the composition required for forming the desired tissue is implanted in the patient at a desired location a bud is formed which grows via morphogenesis into the desired tissue. The specification at page 39, Example 3, also describes obtaining germinal cells (stem cells) from a patient's soft periodontal tissue, which "contain active growth and transcription factors," for promoting growth of any "tooth germ [bud]." A tooth is a multiple tissue organ which includes blood vessels, nerves, etc. A skilled person having experience in the medical arts reading the instant specification would understand that the formation of a bud/ primordium is the foundation for human organogenesis. In this regard, see ¶5 of the Declarations of Drs. C. Gene Wheeler, Wayne H. Finley, and Andrew E. Lorincz (all of record). It is clear that one having experience in the medical arts reading the specification would understand that it teaches an embodiment comprising forming a primordium as a precursor in growing

both soft and hard tissue (see ¶7 of the above-mentioned Declarations of Drs. C. Gene Wheeler, Wayne H. Finley, and Andrew E. Lorincz.) This is consistent with the disclosure at page 31 wherein the specification describes the term "bud" as designating a partially grown tooth. Moreover, it is clear from said Declarations and the specification as a whole that Dr. Elia recognized that stem cells are biological building blocks that promote the growth of cells/tissues in the body.

Finally, the question/issue of whether instant claims 403-405 and 407-412 are definite within the purview of the second paragraph of Section 112 does not depend on their relationship with the scope of claims in co-pending application Serial No. 10/179, 389. The subject matter of the instant claims is defined by language requiring that the implanted composition forms a bud via morphogenesis along predetermined genetic pathways, which bud grows into the desired soft tissue thus providing the desired functional outcome. All that is required by the second paragraph is that the claims define the intended invention with a reasonable degree of clarity. Appellant submits that a reasoned reading of the claim language in light of the instant specification compels a conclusion that the claims satisfy the definiteness requirement of the Statute.

Accordingly, Appellant does not believe that it is necessary to amend the claims as suggested by the PTO at page 3 of the Rejection to overcome this rejection. However, should the Board believe that such amendment is required to overcome the rejection, Applicant stands ready to make the suggested amendment.

Rejection of Claim 404 under 35 U.S.C. §112, First Paragraph - Description

Claim 404 stands rejected under 35 U.S.C. §112, first paragraph for failure to satisfy the "written description" requirement. Specifically, the PTO maintains that the specification fails to provide an adequate description of the limitations commensurate with the specific scope of protection sought by the claim in issue – that the specification fails to *in haec verba* describe "…growing an artery by administration of stem cells to a damaged site in a leg of a patient…"

The Rejection, in particular, points out that no claim reciting "stem cell" and "artery" is rejected for lack of description. The PTO apparently concedes that the specification generally describes the general inventive concept of injecting stem cells for growth of an artery, and for good reason. The PTO rejection admits that pages 47-48 of the specification describes stem cells as an example of a patient's own cells that are contemplated for producing an artery via direct differentiation and morphogenesis as a desired functional outcome. It is also noted that the PTO has not challenged Appellant's position that Example 18 of the specification describes a method of growing an artery in the leg of a patient by injecting a composition which is described in the specification as belonging to a class of compositions that promote soft tissue growth. It is clear that this example describes every limitation called for by claim 404 except the use of stem cells as the injectable composition for promoting the desired soft tissue growth.

The standard for written description applied by the PTO in rejecting claim 404 is tantamount to requiring the specification establish literal support for the claimed combination of features. It is trite law that a specification need not set forth a distinct embodiment corresponding to every claim at issue. Cf. <u>Union Oil of Cal. v. Atlantic</u>

Richfield Co., 208 F3d 989, 997, 54 USPQ 1227, 1232-33 (Fed. Cir. 2000). It is enough for the purposes of the written description requirement of Section 112 of the Statute that the specification contain an equivalent description of the claimed subject matter sufficient to convey to one having experience in the medical arts that applicant was in possession of the claimed invention, i.e., locally injecting a class of artery growth promoting compositions encompassing a patient's own stem cells at a desired site in the patient's leg. See in particular Lockwood v. American Airlines, Inc., 107 F3d.1505, at 1572, 41 USPQ 2d. 1961 (Fed. Cir. 1997).

The PTO's Ruschig analysis is inapt because that case involved a phantom claim copied from another inventor, which did not find descriptive support in Ruschig's application. The claim at issue is drawn from Appellants specification itself which describes a class of compositions for promoting the growth of soft tissue, which broadly includes cells and more specifically bone marrow stem cells. The specification at page 37 teaches that cellular products are growth factors. The specification also discloses using a patient's own bone marrow as a source of autologous stem cells for promoting growth of an artery via differentiation and morphogenesis. Page 44, lines 22-24 of the specification contemplates using VEGF "or its growth factor equivalent" for promoting artery growth. It is clear that all the claimed limitations appear as words in the specification. Accordingly, the necessary blaze marks directing one having experience in the medical arts desiring to practice the protocol of Example 18 to inject a cellular growth factor, such as a patient's own stem cells, as an alternative composition for promoting artery growth in a patient's leg exhibiting vascular damage is clearly provided by the instant specification.

The PTO argues that while the disclosure on pages 47 and 48 of the specification teaches using stem cells (e.g., autologous stem cells) for growing an artery among other organs, it does not describe treating a damaged site in a leg. A person of experience in the medical arts reading the specification would understand that growing an artery in the leg of a patient is one of the desired functional outcomes of the invention described in the specification. Aside from the written description set forth in the specification, skilled medical practitioners, Drs. Richard Heuser and Andrew E. Lorincz, declared that the specification taught them that the described administrative techniques, including injection, would be useful for implanting the growth factor compositions disclosed in the specification, such as stem cells, for growing an artery in a patient. See ¶5 of the Declaration of Dr. Heuser and ¶6 of the Supplemental Declaration of Dr. Lorincz (both of record).

At ¶10, pages 8 and 9 of the Rejection, the PTO questions whether one experienced in the medical arts reading the specification would understand that Appellant's usage of the term growth factor was intended to include compositions comprising genes and bone marrow stem cells. Lest there be any doubt whether the answer is positive, one need look no further than ¶¶5-7 of the Declarations of Drs. Wheeler, Finley, and Lorincz, ¶6 of the Declaration of Dr. Heuser, the Supplemental Declaration of Dr. Lorincz, and the definition found in the Alberts et al. publication definition of "growth factor" cited and made of record by the present PTO Examiner in co-pending Application Serial No. 09/836,750 and entitled, Molecular Biology of the Cell, 4th Ed., Chapter 17 (attached hereto as Exhibit A and hereinafter "Alberts"). Alberts' definition of a growth factor is consistent with Appellant's definition found on

page 43, lines 18 and 19 of the specification, "Growth factors control cell growth, division, differentiation, migration, structure, function, and self-assembly." Moreover, the PTO, in making restriction requirements prior to and subsequent to the date of the instant Rejection, has consistently identified genes and cells as species of the genus "growth factor". The present PTO Examiner apparently has decided to not accord full faith and credit to such PTO determinations. Accordingly, Appellant is mystified by the present PTO Examiner's insistence upon burdening the record with petty issues that were thoroughly vetted previously by the PTO, via Petition to the Commissioner in the instant application, and subsequently followed by the PTO.

The PTO, by applying strict rules derived from a misapplication of legal precedents, is attempting to use the written description requirement to eviscerate a claim narrowed during prosecution based on a broader disclosed invention. The court in Ralston Purina Co. v. Far-Mar-Co, Inc., 772 F.2d 1570, 227 USPQ 177 (Fed. Cir. 1985) noted that a combination of features in a claim need not exactly respond to those in the specification- the issue is whether one of skill in the art could derive the claimed combination from the specification. All that is required is that the specification provide enough disclosure to show that Appellant "invented what is claimed." See Vas-Cath, Inc. v. Mahur-kar, 935 F.2d 1555, 1563-64, 19 USPQ2d 111, 1117 (Fed. Cir. 1991). Written description determinations are fact-sensitive and should be dealt with on a case-by-case basis without adhering to strict rules. Also see In re Wertheim, 541 F.2d 265, 191 USPQ 90, 96 (CCPA 1976) wherein the court held that the claimed combination did not have to find verbatim support in the specification. Appellant believes that the Ralston Purina and Wertheim decisions govern this case. Appellant submits that the rejection should be

reversed because the record shows substantial evidence of adequate written disclosure for claim 404.

Rejection of Claims 403-405 and 407-412 under 35 U.S.C. §112, First Paragraph – Enablement

Claims 403-405 and 407-412 stand finally rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the enablement requirement. Appellant responds to the rejection of claims 403-405 and 407-412 in the following three sections, wherein patentability is argued separately in each section.

Rejection of Claims 403, 411, and 412 under 35 U.S.C. §112, First Paragraph – Enablement

Claims 403, 411, and 412 stand finally rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the enablement requirement. Appellant disagrees that the subject specification fails to enable the claimed subject matter under current law. Appellant herein argues the patentability of each claim.

It is well settled that enablement issues are determined by consideration of an applicant's specification along with knowledge in the art at the time of filing, United States v. Telectronics, 857 F. 2d 778, 785; 8 USPQ 2d 1217, 1223 (Fed. Cir.1988, *cert. denied* 490 U.S. 1946 (1989). Appellant submits that the instant specification, when considered in view of the knowledge in the art at the time the application was filed, enables one skilled in the medical art to make and use the claimed invention.

Appellant submits that there are three major points to consider when determining whether the instant specification contains a disclosure that would have enabled a skilled

person in the medical art to make and use the claimed invention within the purview of the statute. The points are: 1) the specification disclosure; 2) the knowledge in the art at the time the application was filed; and 3) the skill level in the art. When these points are considered, there should be no doubt that Appellant's specification provides an enabling disclosure.

As to the first point, there is a considerable body of disclosure provided by the subject application relating to Appellant's disclosed invention of promoting the growth of soft or hard tissue in human patients—including growing a new artery as called for by the claims at issue—by administering a broad class of growth factors, including stem cells, suitable for effecting such tissue growth. Note that Appellant's specification (pages 10, 20, 21, 30-33, and 37-52) provides a substantial body of disclosure regarding using a growth factor to form a bud and thus grow soft tissue in a human body. These portions of the specification describe a class of claimed and unclaimed growth factors that broadly and specifically include genes, nucleic acids, a patient's own cells (autologous cells), or universal cells, e.g., stem cells (global mononuclear bone marrow cells), etc., all of which are described to promote tissue growth through differentiation and morphogenesis. Appellant's broad and specific disclosure relating to the aforementioned class of growth factors patently provides a scope of enablement which includes stem cells broadly (pages 37, 48, 50, and 51) and bone marrow mononuclear stem cells specifically (pages 40-42). Such disclosure is commensurate in scope with the subject matter of the claims at issue.

As to the second point, the record clearly establishes that the administration techniques, apparatus, and administered compositions disclosed and claimed by Appellant were old and well known as of the filing date of the instant patent application.

U. S. Patent No. 5,980,887 to Isner et al. (hereinafter "Isner" and of record) and the Asahara et al. publication, cited in Isner, published in <u>Science</u> and entitled "Isolation of Putative Progenitor Endothelial Cells for Angiogenesis" (hereinafter "Asahara" and of record) constitute contemporary prior art knowledge which employed a limited subpopulation of EC progenitor stem cells isolated from human peripheral blood for promoting capillary growth. Isner and Asahara are evidence that those skilled in the art prior to the Appellant's 1998 filing date were aware that EC progenitor cells (stem cells) and DNA encoding VEGF are alternatives for treating blood vessel injuries, i.e., ischemic tissue. Isner, at column 7, lines 17-23 of the patent, discloses that "any suitable means" can be used to administer stem cells, including intramuscular injection. U.S. Patent No. 5,328,470 to Nabel et al. (hereinafter "Nabel" and of record) teaches one skilled in the art that cells and genes can be either locally (injection) or systemically administered to human patients to treat organs affected by disease, including ischemic tissue. Although these patents are directed to different inventions than that of Appellant, i.e., employ different cells and achieve different results, they nevertheless apprise one skilled in the art of prior art methods commonly used for administering genes and cells for the treatment of human diseases involving ischemic tissue. Such objective evidence must be taken into consideration by the PTO when determining enablement under 35 U.S.C. §112, first paragraph.

One skilled in the art reading the instant specification's teaching of using stem cells harvested from the bone marrow or blood of the patient would understand that the claimed invention distinguishes from Isner by describing using unfractionated (global) bone marrow mononuclear cells and in achieving a different functional outcome. There is

no basis in fact for determining that a fractionated population, such as EC progenitor cells, is disclosed by Appellant because there is no disclosure that the harvested cells are separated and then a separated portion administered to a patient. Reading the disclosure otherwise distorts the reasonable/intended reading of Appellant's specification. Isner serves to apprise one skilled in the art of general methods for implanting endothelial progenitor stem cells for forming capillary blood vessels and restore the endothelial lining of blood vessels. One skilled in the art being so apprised and reading the instant specification would understand that Appellant has provided sufficient information, i.e., the process steps and ingredients essential to grow an artery as set forth in the claims.

Further evidence supporting enablement may be found in the form of the February 13, 2001 Declaration of Dr. G. Robert Meger (of record) which demonstrates that the disclosed and claimed administration techniques used in practicing the invention were known at the filing date of the application. The administration techniques disclosed by Appellant were routinely employed in the medical art, but not in the claimed combination with the claimed materials, at the time the instant application was filed. See in particular the discussion in Isner and Asahara in regard to the medical art's prior use of bone marrow transplants (HSCs) in treating diseases. Isner acknowledges using techniques similar to those used in the medical arts for recovering HSCs in obtaining endothelial progenitor cells (CD34+). The collection, handling, and reimplantation of HSCs are so well known and notorious in the medical arts that the Board should take Official Notice of same.

In any event, Appellant submits that such disclosure of the instant specification and existing knowledge in the art, such as that identified by Dr. Meger, as well as the

work of Isner, Asahara, and Nabel, would enable a skilled practitioner to practice the claimed invention. As will become evident later, two experts in the medical field, Drs. Richard Heuser and Andrew E. Lorincz, being apprised of relevant portions of Appellant's specification, confirm such conclusion.

As to the third point, the PTO has acknowledged that the level of skill in the medical art is high. Appellant agrees that the skill level is high when it is considered that many years of education, training, and experience are required in the medical field. The instant specification is thus addressed to, and consequently would be understood by, such highly skilled persons.

Once the above-identified relevant materials and administration techniques set forth in the subject specification are properly considered in their entirety, Appellant believes that there should be no question that one skilled in the medical art is enabled to make and use the claimed invention. This conclusion is reinforced, as noted above, by the fact that the materials and administration techniques, but not the inventive results, were well known when the instant application was filed. MPEP Section 2164 states that the purpose of the enablement requirement is to describe the claimed invention in such terms to permit one skilled in the art to make and use the invention. Such Section cautions that detailed procedures for making and using the invention may not be necessary if the description of the invention itself is sufficient to permit those skilled in the art to make and use the invention. For the reader's convenience, MPEP Section 2164.01 states that:

> A patent need not teach, and preferably omits, what
> is well known in the art. *In re Buchner*, 929 F2d. 660, 661,
> 18 USPQ 2d 1331, 1332 (Fed. Cir. 1991); *Hybritech, Inc. v.*
> *Monoclonal Antibodies, Inc.*, 802 F2d. 1367, 1384, 231

USPQ 81, 94 (Fed. Cir. 1986) cert denied, 480 U.S. 947 (1987); and *Lindemann Maschinenfabrik GMBH v. American Hoist and Derrick Co.*, 730 F2d. 1452, 1463, 221 USPQ 481, 489 (Fed. Cir.1984).

Appellant believes that the above caution is especially relevant to the instant factual situation where the Examiner has conceded that there was a high level of skill in the art at the time the instant application was filed and further in view of the evidence contained in Isner, Asahara, Nabel, and Dr. Meger's Declaration that the methods and apparatus needed to practice the invention were well known at the time of the invention. Thus, Appellant submits that the instant disclosure clearly enables one skilled in the medical art to make and/or use the full scope of the claimed invention without undue experimentation. Any reasonable consideration of the three above-delineated points and the interaction thereof compels such conclusion.

Appellant's above conclusion that one skilled in the art is enabled to make and use the claimed invention is consistent with the PTO's acknowledgement at page 14, ¶18 of the Final Rejection of May 5, 2008 that the state of the art after the Isner disclosure was such that Appellant's claimed "…method was known to be possible." (of record and attached hereto as Exhibit B for the readers' convenience). Accordingly, the enablement issue should be put to rest because the Isner and Asahara disclosures are prior to, or contemporary with, the filing date of the instant application.

The PTO has the burden to establish and support by convincing objective evidence a *prima facie* case of lack of enablement. For reasons set forth below, Appellant believes the PTO has failed to meet such burden.

The first paragraph of the statue requires nothing more than objective enablement, and it is of no importance whether such teaching is set forth by use of illustrative examples or by broad terminology. As a general matter, an application's disclosure, which contains a teaching of how to make and use the invention in terms which correspond in scope to those used in describing the invention sought to be patented, is considered to be in compliance with the enabling requirement of the statue. In re Marzocchi, 439 F.2d 220, 169 USPQ 367, 369-370 (CCPA, 1971). Further, "Section 112 does not require that a specification convince persons skilled in the art that the assertions therein are correct." [Emphasis added]. In re Robins, 429 F.2d 452, 166 USPQ 552 (CCPA, 1970).

Turning to the reasons proffered by the PTO regarding non-enablement, Appellant presents the following remarks.

The PTO, at pages 10 and 11 of the Rejection, stated that, "The rejection of record has found that *the breadth of the claims* and *the amount of direction and guidance present and the presence or absence of working examples* are the principle [sic] factors that speak against the enablement for the claims under consideration." These factors are discussed below in rebuttal to the lack of enablement rejection.

When evaluating enablement, it is incumbent upon the PTO to determine what subject matter each claim recites, i.e., the scope of protection sought for each claim. The scope of dependent claims are properly determined with respect to 35 U.S.C. §112, fourth paragraph. See MPEP Section 2164.08. It is submitted that the PTO has failed to perform such required analysis. Appellant notes that the PTO has not addressed the subject matter of each claim separately, but instead at page 11, ¶13 of the Rejection asserted that the

elements that are essential and common to all of the claims are not enabled by the disclosure. However, beyond such general assertion, the PTO has not explained why stem cells harvested from bone marrow (claim 407) or stem cells harvested from blood (claim 409) of from the patient (claims 408 and 410) are common to the generic term "stem cells" in the sense of enablement. Appellant has argued that the subject matter of all claims finds enabling support in the specification.

Appellant further points out that it is evident the PTO failed to consider the disclosure provided by the subject specification as a whole in determining compliance with the enablement requirement of the statute. The appropriate factual determination is whether the instant specification reasonably directs one skilled in the art how to make and use the claimed subject matter. The PTO erroneously restricted the factual determination to the elected species of growth factor and, thusly, ignored those portions of the specification describing a broader generic invention and also ignored disclosure related to non-elected species. Appellant is entitled to have the entire disclosure considered in determining compliance with 35 U.S.C. §112, first paragraph. See In re Anderson, supra and In re Johnson and Farnham, supra and such determination must take into consideration that which is known in the prior art–that a patent should preferably omit that which is well known/understood in the particular art to which the claims are directed.

The PTO at pages 11-15, ¶¶14-16 of the Rejection, asserts that the specification does not disclose with specificity which cells would or would not work for growing an artery. The PTO's above assertion that an applicant must disclose with specificity which cells would not form an artery is erroneous. An applicant is required to provide information that would enable one skilled in the art to which the invention pertains to

make and use the claimed invention and is not required to provide information that would not enable one skilled in the art to make and use such invention; i.e., to form an artery.

In any event, the PTO has alleged that the term "stem cells" encompasses a large subgenus of cells and thus somehow does not advise the skilled person which stem cells to use or not to use. When one reads Appellant's specification with the eyes and understanding of a person skilled in the medical art, there should be no question that such person is informed as to the types of stem cells that grow multiple tissue organs, such as an artery. To grow an artery one must use pluripotent stem cells because growth of an artery comprises multiple tissues including muscle and endothelial tissue. Those skilled in the art recognize such elementary fact. The specification teaches on pages 40-42, 47, and 48 utilizing autologous stem cells harvested from bone marrow and blood of the patient (self-cell therapy) or from cell cultures (allogenic) to grow organs, i.e., arteries, by differentiation and morphogenesis (page 48). There can be no doubt that the specification teaches that bone marrow cells promote the growth of organs. Further, the specification on page 50 specifically discloses that implanted pluripotent growth factors direct adjacent cells to reconstruct body parts along genetically predetermined pathways. Those skilled in the art would recognize that bone marrow would comprise pluripotent stem cells. In addition, the skilled person is informed by the specification that pluripotent cells, such as some stem cells, are required for growing organs requiring multiple tissues. Moreover, those skilled in the medical art are well aware that bone marrow comprises pluripotent stem cells. In this regard, see the internet article cited by the present PTO Examiner in the Rejection published in The Journal of Invasive Cardiology, Vol. 17, July 1, 2005, entitled "Progenitor Cell Transplantation and Function following Myocardial Infarction."

(of record and attached hereto as Exhibit C). Appellant attaches the complete article because the present PTO Examiner did furnish a complete copy, and the referred-to passage was omitted from the copy furnished by the present PTO Examiner. Accordingly, one skilled in the art reading the subject specification, would understand that all that is required to practice the claimed invention is to use pluripotent stem cells, such as those disclosed in the instant specification, to grow an artery.

Turning more specifically to the PTO's allegation that the subgenus "stem cells" is large and thus does not advise the skilled person which stem cells to use or not use, Appellant points out that the specification describes the subject matter of claims 407-410, stem cells harvested from bone marrow and stem cells harvested from blood, at pages 40–42, and pages 47–52 wherein the specification discloses using a patient's own stem cells for growing multiple described organ species through differentiation and morphogenesis. Furthermore, the organ species artery is specifically disclosed as a desired target organ on page 52 and, as stated previously, pluripotent stem cells are described at page 50. Such disclosure, along with the functional claim requirement that an artery is grown, is believed to be sufficient to support the subgenus stem cells.

As noted above, the PTO appears to erroneously require that Appellant also disclose specific types of stem cells that do not form multiple tissue organs, i.e., that are unipotent, rather than pluripotent. Unipotent cells are well known and characterized in the medical art (and would be identified by a skilled person). Illustrative of unipotent stem cells of record in the instant application or of record in related applications include: the progenitor epithelial cells of Isner; the skeletal muscle cells of the Murry et al. 1996 publication in J. Clin. Invest. entitled, "Skeletal Myoblast Transplantation for Repair of

Myocardial Necrosis" cited by the Examiner in the November 28, 2003 Office Action in co-pending application Serial No. 09/836,750 and attached hereto as Exhibit E; and the mesenchymal cells of Caplan et al. (of record). Workers skilled in the medical arts are aware that unipotent stem cells are specific in that they are limited to promoting growth of a single tissue type, e.g., endothelium, and are incapable of promoting the growth of multiple tissue organs. It is submitted that the PTO has applied an unreasonable standard by requiring that the specification identify inoperative members of the subgenus "stem cells" in order to comply with the enablement requirement.

The PTO has also raised the issue that Appellant's disclosure does not teach the use of unfractionated (unfiltered) bone marrow mononuclear cells. Such issue is believed to be specious because there is simply no disclosure in the subject specification which would direct one skilled in the art to use a separated composition of mononuclear cells harvested from bone marrow other than an unfractionated one. The failure of the specification to teach separating and excluding any given fraction of mononuclear bone marrow stem cells is consistent with the requirement for using an unfractionated bone marrow composition and constitutes a reasonable reading of the specification. See Phillips v. AWH., Corp., 415 F. 3d 1303, (Fed. Cir. 2005). Hence, one skilled in the art would understand that the claimed stem cells harvested from bone marrow refer to an unfractionated population.

The PTO also alleged that the expressions "stem cells harvested from bone marrow" and "stem cells harvested from blood" were typically understood to refer to the CD34+ fraction. One only has to note that the post-filing date Strauer et al. publication in Circulation entitled, "Repair of Infarcted Myocardium by Autologous Intracoronary

Mononuclear Bone Marrow Cell Transplantation in Humans" and (hereinafter "Strauer" and of record) and U.S. Patent No. 7,097,832 to Kornowksi et al. (hereinafter "Kornowski" and of record) had no such misunderstanding of the term "stem cell." Moreover, one skilled in the medical art would also be well aware of the medical art's widespread use of bone marrow transplants for treating disease. Such transplants utilize unfractionated (the entire array of bone marrow stem cells) compositions. As pointed out earlier, one skilled in the art would understand that the term "stem cells harvested from bone marrow" refers to the entire population, not to an unspecified fraction thereof. It is noteworthy that Isner, as would any other competent medical practitioner, was careful to disclose that the stem cells of his invention were separated from the entire population and then characterized.

In the previously mentioned complete internet article entitled, "Progenitor Cell Transplantation and Function following Myocardial Infarction," Dr. O'Neil acknowledges that there are two schools of thought as to which cells to use–unfiltered bone marrow (Appellant, Strauer, and Kornowski) or CD34 positive cells (Isner). In any event, one skilled in the art reading the instant specification's teaching of using stem cells harvested from the bone marrow or blood of the patient would understand that the claimed invention distinguishes from the CD34+ fraction of Isner by describing using unfiltered (global) bone marrow mononuclear cells. As pointed out earlier, there is no basis in fact for the PTO to determine that the instant specification provides guidance to one skilled in the art for implanting anything other than the entire array of bone marrow derived cells harvested from the patient's bone marrow or blood. Whether one uses the terms "global," "whole population," "unfiltered," or "unfractionated," matters not a whit.

Certainly, unlike Isner, the concept of isolating/separating of a component of the entire array of bone marrow stem cells is not implicitly or explicitly contemplated or described in the instant specification or in Strauer and Kornowski. It is clear from such disclosure what Appellant intended the term to mean, and the claims on appeal must be interpreted accordingly. See Phillips v. AWH., Corp., supra. Reading and interpreting the disclosure otherwise is improper because it distorts the reasonable meaning provided to one skilled in the art by Appellant's specification. The PTO's attempt to interpret the specification to limit stem cells to a cell population less than the whole is akin to reading new matter into the specification.

The PTO at pages 15 and 16, ¶¶17 and 18 of the Rejection, asserts that the instant specification fails to provide any guidance as to how to use any kind of cell, much less a stem cell, to grow an artery. The above statement of the PTO appears to have overlooked that the claims on appeal require that the cells are stem cells. In any event, the disclosure at page 47, line 22 through page 48, line 15 of the specification clearly rebuts the PTO's notion that Appellant never clearly enunciated using stem cells (harvested from bone marrow and blood) for promoting direct differentiation and morphogenesis into an organ. Of course as admitted by the PTO, one skilled in the art would recognize that growth of an organ encompasses an artery. In any event, page 45 of the specification sets forth the well recognized medical fact that "[a]n artery is an organ from the circulatory system." PTO's statement that the specification fails to "provide any guidance as to how to use stem cells to grow an artery" evinces a lack of understanding of how differentiation and morphogenesis occurs *in vivo*. No guidance is necessary because the art skilled would recognize that once the stem cells are implanted, artery growth proceeds along

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genetically predetermined pathways. The fact that stem cells home to foci of ischemic tissue was known to those skilled in the art at the time of filing of the instant specification, as evidenced by Asahara. The PTO's position that Appellant has asserted contradictory weight to Isner is not understood. In any event, the answer to the PTO's question as to how to grow an artery is remarkably simple and is reflected by the claims on appeal. One simply locally places a stem cell of the type capable of forming an artery, such as by intramuscular injection, and the body forms a bud which then grows into an artery. By disclosure, not at the direction of Isner as alleged by the PTO, "locally placing" means placing at or adjacent the site where artery growth is desired. Certainly, minimal guidance is required by one skilled in the art to perform such a notoriously old administrative procedure.

The PTO, at pages 17-22, ¶¶19-23 of the Rejection, addresses Appellant's argument that the entire specification disclosure has to be considered in determining whether the enablement requirement of Section 112 has been satisfied. As recognized in the last sentence of ¶23, the PTO is charged with reading the "actual content" of the specification when rendering such determinations. Appellant maintains that a person of experience in the medical arts reading the actual content of the specification beginning at line 22, page 47 through line 15, page 48 would understand that its context contemplates reimplanting a patient's own stem cells to grow an artery as a desirable functional outcome. The PTO charges at pages 17-18, ¶20 of the Rejection that Appellant has taken language from different portions of the text in order to support the claimed language. Apparently, the PTO originally failed to consider the following two paragraphs from page 48 of the specification:

During reimplantation one of the patient's own cells is returned to the patient. During implantation, a cell not originally obtained from the patient is inserted on or in the patient.

In the example above, if germinal cells (and in some cases, stem cells) are utilized a direct differentiation and morphogenesis into an organ can occur in vivo, ex vivo, or in vitro.

When read in its proper context by one skilled in the medical art, the language on page 48, line 13 of the specification "[i]n the example above..." refers to the formation (page 47, line 22) of "[o]rgans and/or tissues...formed utilizing the patient's own cells." Only an unskilled person in the medical art would be confused by the disclosures on pages 47 and 48. The PTO rejection at page 19, ¶21 apparently agrees that Appellant's interpretation is reasonable.

Appellant disagrees with the PTO's position that even if Appellant's interpretation of the specification is reasonable, it does not teach one skilled in the art how to use stem cells for growing an artery. The specification teaches that the reimplanted stem cells effect organ growth via direct differentiation and morphogenesis (page 48, lines 13-15). The specification (page 45, lines 1-4) contemplates growing an artery in the "heart, legs or other areas." The specification contemplates using bone marrow aspirant harvested from a patient (pages 40–42) as a potential source of stem cells used for the promotion of organ growth. During reimplantation, stem cell aspirant obtained from a patient's bone marrow is returned to the patient by injection at a desired location to promote the growth of an artery via direct differentiation and morphogenesis. Bone marrow transplant therapies have been in practice for decades. It is unnecessary for the specification to provide a detailed explanation of how one would go about obtaining

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autologous stem cell aspirant because experienced persons in the medical arts are well aware of such a procedure. Protocols for safely handling and processing such stem cell aspirant are well known and have been in practice for decades. Thus, the instant specification presents one skilled in the art with sufficient information how to obtain stem cells from a patient and how to administer these stem cells to the heart, leg, or other desired site in order to promote artery growth. Once implanted, the autologous stem cells promote growth of an artery via direct differentiation and morphogenesis along genetically predetermined pathways. As pointed out earlier, persons skilled in the medical arts are well aware of the role of stem cells in organogenesis. Appellant submits that the specification clearly enables one skilled in the medical arts to select and obtain bone marrow aspirant from a patient and to reimplant such aspirant at a desired site in said patient to promote artery growth via direct differentiation and morphogenesis along genetically predetermined pathways.

The comments in ¶¶22 and 23 of the Rejection evince a misunderstanding of Appellant's criticism of the PTO's propensity for referring to portions of the specification relating to unclaimed inventions. While it is true that it is proper for the PTO to review the whole specification in determining compliance with the first paragraph of 35 U.S.C. §112, Appellant believes that it would be more expeditious and economical on all parties to focus on portions of the specification relevant to subject matter of the claims in issue in order to develop succinct issues for appeal. Appellant believes that such practice unnecessarily results in an engorgement of the record on appeal and adds little, if any, in the way of evidence to the PTO's case in chief.

The PTO, at pages 22-27, ¶¶24-27 of the Rejection, addresses the question of extrapolating dosages of VEGF cDNA to cell dosages. Appellant disagrees with the PTO's position. Initially, Appellant submits it is clear from MPEP Section 2164.01(c) that it is not necessary to specify the dosage if one skilled in the art could determine such information without undue experimentation. The PTO apparently acknowledges, at page 22, ¶24 of the Rejection, that there is no enablement issue regarding the absence of guidance as to how many stem cells should be used to grow an artery. Appellant concurs with such a conclusion. The PTO states that Appellant's dosage extrapolation is only under discussion "…because Appellant apparently seeks to establish that an extrapolation of this type is so well known in the art that it would be implicitly understood to be present in Example 18 of the specification."

Appellant never argued that an extrapolation of this type is so engrained in the prior art that it is necessarily implicit in Example 18. While Appellant agrees that dosage is not an enablement issue, nonetheless the following comments in regard to the calculus employed in the conversion is proffered in an attempt to present a complete rebuttal even at the expense of engorging the instant Brief.

Appellant used a well established weight basis conversion method employed in the medical art for decades to convert the gene dosage of Example 18 to cells. Appellant never argued the viability of conversion across all species lines. Appellant's extrapolation was originally proffered in response to previous PTO criticisms regarding dosages and was presented to demonstrate if necessary how one skilled in the medical art could easily and routinely convert the gene dosage described in Example 18 to cell dosage on a mass basis. Use of such conversion based on mass is believed to be valid in this case because

one skilled in the art would reasonably understand from reading the subject specification that Appellant was in possession of the concept that genes and cells are equivalent compositions for growing soft tissues in a body. The conversion was suggested solely for the purpose of illustrating that one skilled in the art desiring to employ stem cells as a VEGF growth factor equivalent in Example 18 could readily use the dosages of cDNA clones to obtain approximate equivalent cell dosage based on weight.

The PTO's statement that it is untrue that the medical art has used such conversions for the past fifty years in cell therapy because they "would not recognize the terminology or even imagine the concept of conversion depicted in Exhibit D" eschews a want of factual basis. The PTO rejection is devoid of any objective evidence supporting such a position. It is also apparent that Drs. Heuser and Lorincz disagree. Need the PTO be reminded that Dr. Heuser is a preeminent cardiologist associated with Bioheart in the field of cell therapy and that Dr. Lorincz has many years of experience in the medical art? In any event, both Drs. Heuser and Lorincz are quite experienced in dosage practice.

The PTO Rejection at page 24, ¶25, based on an esoteric presentation of differences between plasmid DNA and genomic DNA found in cells, attempts to explain why "it is fundamentally illogical to equate recombinant plasmid DNA to cellular DNA on the basis of mass." However, the PTO has not sufficiently explained why the reasonableness of using such weight conversion appears to be supported by the fact that such converted dosages are commensurate with those used by workers in the art using bone marrow stem cells to grow an artery, such as that reported in Strauer. The PTO alleged, at page 29 of the Final Rejection of May 5, 2008, that the above correspondence of dosages with Strauer was "pure coincidence" and that Appellant "stumbled upon" a

simple method for determining cell numbers. It is clear from such unfounded characterization that the PTO has paid no deference to the conversion practice used routinely for decades by the medical art. Regarding the alleged "pure coincidence," further attention is directed to the gene and cell dosages of Isner at column 11, lines 4-9 and column 7, lines 17-23, respectively. A conversion of the dosages of nucleic acids of Isner to corresponding dosages of cells was conducted.[1]

It is evident from the conversion of nucleic acid dosages to cell dosages that the converted cell dosages fall within the range specified by Isner. The reasonableness of the conversion has been previously demonstrated regarding a conversion of the dosage of Example 18 in the instant application to the bone marrow stem cell dosages specified by Strauer. Hence, the usefulness of the well-known and established weight conversion has been attested to and demonstrated in two diverse cases. Appellant believes this fact constitutes compelling and unchallenged evidence that the PTO's criticism of the conversion is unwarranted. The Third Supplemental Declaration of Dr. Richard Heuser (of record and originally filed in co-pending application Serial No. 10/179,589) and the Second Supplemental Declaration of Dr. Andrew E. Lorincz (of record and originally filed in co-pending application Serial No. 10/179,589) confirm that the use of such well known tool is reasonable in the medical art. Accordingly, Appellant believes that the PTO's above comments are based upon unsupported speculation and opinion rather than upon objective evidence.

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[1] Isner specified a common dosage of 2000 micrograms for the more preferably and most preferably dosage ranges. Such common dosage was utilized in the conversion calculations. The weight of nucleic acids of an average cell was considered to equal 40 picograms (pg). The 2000 microgram dosage was converted to pg by multiplying by 10^6 equals 2000 x 10^6 pg. An average weight of 40 pg was used for nucleic acids as consistent with the prior conversion. The conversion was then made by dividing 2000 x 10^6 by 40 to arrive at a cell dosage of 50 x 10^6 and falls with in the range specified by Isner.

The PTO, at page 25, ¶26 of the Rejection, incorrectly asserts that Appellant based the extrapolation calculation on the teaching of Isner. It did not happen that way; nor did Appellant assert that Isner reported a link between plasmid DNA dosage and cell dosage. All that Appellant pointed out was, like in the case of Strauer, the conversion of plasmid DNA to cells was overlapping in regard to dosages. The PTO is exercising clairvoyance in speculating that Isner did not base his cell dosages on previous work with genes. Only Isner and his coworkers know the answer to that question. The PTO has not challenged this fact except to assert that it is coincidence. Such incorrect determination formed the basis for the equally incorrect conclusion that gene therapy and cell therapy have different status in the art and, therefore, cannot be considered as functional equivalents of one another. One need look no further than Isner and Asahara to dispel such erroneous opinion. One can gather from the comments that the PTO is aware of the large doses of cells used in cell therapy, which further mitigates any notion that dosages are critical. It is well known in the medical arts that large doses of stem cells are easily tolerated in patient's undergoing bone marrow transplants without adverse side effects.

The PTO asserts at page 26, ¶27 of the Rejection that Appellant's *post hoc* derivation (extrapolation) is not implicit from any teachings in the specification. Such argument misses the point, which is that such extrapolations have been used for decades in the medical arts in regard to cell therapy and are part and parcel of the prior art. That which is well known in the art need not be included in Appellant's specification in order to comply with the enablement requirement of Section 112, first paragraph. See MPEP Section 2164.01.

Appellant submits that the dosage extrapolation and the opinions in regard thereto expressed in the Declarations of Drs. Heuser and Lorincz speak for themselves and confirm the reasonableness of Appellant's conversions. Regarding the PTO's statement that no evidence outside Appellant's declaration evidence has been provided, it is of particular note that the extrapolated dosages compare favorably (overlap) with the dosages of global bone marrow cells used by Strauer for treating myocardial infarction in human patients and in Isner for different stem cell populations and soft tissue growth, thereby confirming the reasonableness of the respective Declarants' opinions. Except to allege that such similarities are coincidence, the PTO has not demonstrated or explained why the extrapolation holds true for both Strauer and Isner. Finally, Appellant never argued that Example 18, in and of itself, explicitly suggests using stem cells to grow an artery. What Appellant asserts is that Example 18, when considered with the entire specification disclosure, would lead one skilled in the art to understand that the intramuscular injection protocol set forth therein could be used effectively with any of the described growth factor equivalents, such as bone marrow stem cells, to grow an artery in a patient at a selected site.

The PTO, at page 26, ¶27 of the Rejection, appears to mistakenly believe that the calculus is "Applicant's formula." The PTO's challenge in regard to the technical basis underlying the conversions is misdirected. Such challenge should be directed toward the originators of this well known medical tool and workers in the art who used such alleged faulty calculus—not with Appellant's experts who simply confirmed that the calculus was reasonable and found its roots in the medical art because it is notoriously well known that dosages are commonly specified on a weight basis.

The PTO's *ad hominem* criticism of Appellant's conversion fails to adequately give weight to its evidentiary value. Appellant's evidence establishes as a material fact that physicians have long used conversion charts/formulas for estimating dosages of cells from nucleic acids. It is clear from the record that cell survival and differentiation are not paramount considerations in determining cell dosages because the general practice is to employ multiple doses since stem cell overdosing has not proved to be problematic. Those skilled in the art are aware that safe dose ranges have been established over years of medical practice directed to bone marrow transplant cell therapy. The Board's attention is again directed to the expert opinions of Drs. Heuser and Lorincz, which validate the reasonableness of Appellant's dosage conversions.

On page 27, ¶28 of the Rejection, the PTO states that the specification's failure to report human clinical trials does not constitute the basis for the enablement rejection. It has been Appellant's understanding from the beginning that there is no legal standard requiring either animal or human clinical modeling. Prophetic disclosures are permitted under the rules, statute, and case law. However, the PTO concludes, without further explanation, that the lack of actual examples "contribute significantly," i.e. was a contributing factor along with "other Wands factors," in determining of lack of enablement. It is the burden of the PTO to specifically and precisely point out why the absence of specific working examples, along with any "other Wands factors," supports a *prima facie* case of non-enablement. Appellant submits that the PTO has not met such burden.

On page 27 of the Rejection, the PTO asserts one of skill in the art might surmise from Appellant's specification "...a method to use autologous stem cells to grow an

artery was suggested." This assertion is consistent with pages 34 and 35, ¶36 of the Rejection wherein the PTO acknowledged that, "It is plausible that cells properly described as stem cell (all claims), stem cell harvested from bone marrow (claim 407 and dependents) or stem cells harvested from blood can cause an artery to grow if they are injected locally at a selected site." Having made such statement, the PTO inconsistently attempts to limit such disclosure to merely concept rather than enabling disclosure pertaining to making and using the claimed invention and charges that not a "single enabled embodiment" of the claimed invention is shown. While asserting that actual working embodiments are not required to meet the requirements of Section 112, first paragraph, the PTO finds Appellant's specification lacking by virtue of failing to show a single organ, part of an organ, tissue, artery, or even a bud formed by placing cells in a body. Such a requirement would necessarily require Appellant to demonstrate an actual clinical model. It would appear to Appellant that the PTO is in denial of applying an improper legal standard by requiring an actual working embodiment.

On pages 28 and 29, ¶30 of the Rejection, it is stated that Appellant has missed the point in regard to the citation of Isner and Kornowski as evidence of enabled disclosures dealing with "biotechnological inventions." The statement that the examples in these patents, while not being directed to clinical trials, are not prophetic is not understood, particularly in the case of Kornowski's claims covering human treatment. The point that Appellant was attempting to make is how can the PTO deny that examples in Isner and Kornowski directed to only animal examples somehow, inexplicitly are not considered to be prophetic when applied to human patients. Perhaps, the present PTO Examiner is unaware of the well established fact that many animal experiments are not

replicated when applied to humans. Hence, there can be no question that such patents were considered to be enabled by the PTO despite the lack of working examples directed to human patient, such as those generated during clinical trials.

At page 29, ¶31 of the Rejection, the PTO took issue with Appellant's statement that "Appellant never intentionally, or unintentionally, linked an absence of an art rejection with proof of enablement." In response to the above points, the PTO cited a passage from Appellant's April 30, 2007, communication to the PTO. Such passage does not support the PTO's assertion. What the PTO has failed to appreciate is the manner in which the Court applied the "state of the art" factor in enablement determinations. In re Wands, 858 F.2d 731, 737, 8 USPQ 2d 1400, 1404 (Fed. Cir.1988).

The present PTO Examiner, at pages 30-33, ¶¶33 and 34 of the Rejection, cited two internet articles published in The Journal of Invasive Cardiology, Vol. 17, July 1, 2005, entitled, "Progenitor Cell Transplantation and Function following Myocardial Infarction" and "Tissue Engineering and Interventional Cardiology." In the Rejection, the present PTO Examiner furnished Appellant with copies of the two above-mentioned articles (attached hereto as Exhibits C1 and D1, respectively). Such copies contain less than the complete content of the published articles and lack proper context. Appellant has provided complete copies of the published articles in attached Exhibits C and D, respectively for the Board's consideration and comparison to the version furnished by the present PTO Examiner.

The two above-identified articles (Exhibits C1 and D1) are relied on by the PTO to challenge Appellant's assertion of post-filing success for the claimed method. The PTO contends the furnished excerpts show that some seven years after the filing date of

the instant application, skilled workers in the art voiced concerns about cell choice, dosages, time of treatment, implantation apparatus and cell survival were unanswered. Initially, Appellant's comparison of the full text of the articles with the versions furnished by the PTO clearly evinces that the present PTO Examiner has artfully selected portions of the context while omitting other portions thereof in an obvious attempt to spin the meaning of the text. Such editing raises a serious question in regard to the probative value of such material as well as the fairness of the administrative process of the PTO.

Appellant has reviewed the limited context from the excerpts presented at pages 30-33 of the Rejection but disagrees that the verbage thereof rises to the level of evidence supporting non-enablement. Most of the comments concerned the BOOST, TOPCARE and Bio Heart trials. The latter body of work is dissimilar from the present invention in that it used a skeletal muscle myoblast product. Dr. Pollman from Guidant Corp. described the BOOST method, "as a simple syringe injection system loaded with 10 cc of bone marrow, 3 cc of which is applied to the coronary arteries." Dr. Pollman does not indicate that any further manipulation was necessary. Appellant has consistently taken the unanswered position that Strauer, relied upon by the PTO, describes little if any experimentation required to practice the disclosed implantation of bone marrow stem cells. Appellant makes the following comments regarding the excerpts presented by the PTO.

- The first quoted statement of Dr. O'Neil is merely asking a question that had been previously answered by Strauer.

- As Dr. O'Neil's second quoted question, neither Dr. Zelher (Guidant, Frankfort) nor Strauer reported any problem with cell hypoxia.

- Dr. O'Neil's third question virtually confirms Appellant's argument that the specification teaches using unfiltered bone marrow.

- Dr. Nikol's comments sound like professional envy rather than critical analysis of bone marrow implantation.

- Dr. Gonschior's comments merely affirm that intravenous infusion would be the simplest method while Strauer's endocardial delivery may be the most efficient. These comments mirror the views expressed by Strauer.

- The quoted comments by Dr. Holmes merely express his criticism of premature human trials and appears to be especially directed to systemic infusion of cells.

Dr. Whitlow's quoted comments are purely theoretical and do not evince that his opinions are based on the performance of any experimental or clinical trials. The autopsy findings described in the Dohmann et al. 2005 publication in <u>Circulation</u> entitled, "Transendocardial Autologous Bone Marrow Mononuclear Cell Injection in Ischemic Heart Failure" cited in the Examiner's Answer dated November 28, 2007 in co-pending application Serial No. 09/794,456 (attached hereto as Exhibit F and hereinafter "Dohmann") show that Dr. Whitlow's theoretical premises are not well founded.

It is puzzling that the PTO can conclude from such selective utterances that "[t]here was a general agreement that more experimentation was needed." This is particularly telling when one understands that the later work of Dohmann and Kornowski closely parallel the work of Strauer and produced similar results.

One final point remains. What is most disturbing to Appellant regarding the PTO's use of these two articles is the omission of information favoring enablement of

Appellant's claimed method. For example, the PTO omitted the statement by Dr. Nikol that, "cells are considered a blood product" and the statement by Dr. O'Neil that, "...because these bone marrow cells are pluripotential..." A further example is the spontaneous utterance of Dr. Heuser that, "[t]he first time I saw this technique presented by the group [TOPCARE] in Frankfort, I was astonished at how simple it actually was," and Dr. Pollman's statement that, "a simple syringe injection system" was used for implantation. Also absent is any reference to Dr. O'Neil's statement regarding "the cascade of processes that actually allow a new cell to come in and regenerate." It is tempting to speculate that the present PTO Examiner's omission of such comments by Drs. Pollman, Heuser, and O'Neil could be attributed to the Examiner's carefully refraining from providing evidence supporting arguments made by Appellant in this and in the companion applications listed in the Related Appeals and Interferences portion of the instant Appeal Brief. In any event, once one skilled in the art realizes that bone marrow promotes the growth of arteries the delivery of the bone marrow is simple. Furthermore, the above utterances indicate that the treatment is not complex as alleged by the PTO. The answer to the PTO's irrelevant question, "Why didn't "[Dr. Heuser] enlighten his colleagues?" is straightforward. Being a patentee in his own right, Dr. Heuser fully comprehends his duty in regard to confidential information, even if the present PTO Examiner is dismissive of such duty. Dr. Pollman, an employee of Guidant, was aware of confidentiality obligations regarding privilege information, as were all of the others. See Dr. Pullman's comment near the bottom of the first page of the "Progenitor Cell Transplantation and Function Following Myocardial Infarction" article (Exhibit C). In addition, an opinion regarding enablement based upon the disclosure of a

patent application is distinct from optimizing medical processes and continuing research involving such processes. The present PTO Examiner's query misses this point.

The PTO, at pages 34 and 35, ¶36 of the Rejection, states that:

> It is plausible that cells properly described as "stem cell" (all claims), "stem cell harvested from bone marrow" (claim 407 and dependents) or "stem cells harvested from blood" can cause an artery to grow if they are injected locally at a selected site.

The present PTO Examiner further characterized the invention to be a series of respectable guesses that later proved true. However, the present PTO Examiner then denied the fact that post-filing references demonstrated that the disclosed and claimed invention confirmed the claimed results. Instead, such post-filing work was credited as being evidence of further experimentation involved in the act of invention. Such characterization is factually inaccurate. Although the PTO did not identify specific post-filing references in the Rejection, the post-filing Strauer publication performs the same steps as claimed and achieves the same results. The record will show that when repeatedly challenged by Appellant to point out where Strauer performed any experimentation, the PTO was not able to identify any such alleged experimentation. Strauer does not describe using any experimental protocol to determine appropriate cell population, i.e., there is no requirement for using a specific subset of bone marrow stem cells. Regarding time of treatment, Strauer does not disclose that determining time of treatment required experimentation. It is clear from the record that the treatment of myocardial infarction (MI) in human patients exhibiting either acute or chronic disease is considered. Strauer elected to treat patients from five to nine days after suffering an MI. Note that in a later 2005 publication in <u>Circulation</u> of Strauer et al. entitled,

"Regeneration of Human Infarcted Heart Muscle by Intracoronary Autologous Bone Marrow Cell Transplantation in Chronic Coronary Artery Disease" cited by Appellant as Exhibit D in an Amendment dated November 21, 2005 in co-pending application Serial No. 09/974,456 (attached hereto as Exhibit G and hereinafter "Strauer 2005") discloses treating chronic MI in patients that had transmural MI some 27 months earlier. Again, no experimentation regarding treatment time was noted. It is evident that the time of treatment following an MI is not a critical variable and undue experimentation would not be required. To the extent that the PTO may be relying on Strauer to establish that the time of administration is critical, Appellant points out that Strauer 2005 is the "best evidence" in regard to whether time of treatment in human patients is critical. Strauer 2005 teaches that stem cells can be used to successfully treat MI in human patients suffering either acute or chronic disease. Moreover, Isner also does not indicate that time is critical in the treatment of humans exhibiting ischemic heart tissue and this was not viewed as an impediment by the PTO. Thus, the PTO's conclusion that unidentified "further experimentation" would be required to practice the claimed invention is not supported on the record and is fatally flawed.

Appellant believes that the above-mentioned lack of experimentation by Strauer actually demonstrates the converse of the present PTO Examiner's hypothesis, i.e., that one skilled in the art would be able to make and use Appellant's so-called "plausible" invention without recourse to experimentation of any kind, let alone an undue amount of experimentation.

As a final point, the PTO, at page 35, ¶37 of the Rejection, refers to the breadth of claims, the amount of direction or guidance, and the presence or absence of working

examples as evidence of that undue experimentation would be required to practice the claimed invention. Although the PTO did not specifically rely upon In re Wands, supra, in this portion of the Rejection, it is assumed that the PTO may have intended to rely upon such decision due to the presented analysis. In In re Wands, the Court focused on three factors: the state of the prior art, the level of skill in the art, and the amount of direction provided by the specification. The specification (pages 47-48) clearly describes the concept of implanting a patient's own cells (autologous stem cells) to promote differentiation and morphogenesis into an organ, which by disclosure includes an artery. The specification teaches numerous methods of implantation including intramuscular injection. The PTO's allegation that the specification fails to address complex problems "that might be encountered" in stem cell therapy is a "red herring," which has not been factually supported on the record. Contemporary prior art wisdom (Isner and Asahara) at the time of Appellant's invention demonstrates the conventionality of intramuscular injection of stem cells and genes in treating disease involving ischemic tissue. Contrary to the PTO's assertion, the post filing work of Strauer does not describe solving any complex problems associated with implanting bone marrow stem cells. Neither the contemporary nor post- filing art disclose any specific problems that had to be addressed and overcome in order to successfully implant cells in a human patient. Thus, the PTO's determination that the specification is non-enabling because it fails to address nonexistent problems is inauthentic.

Appellant believes the instant fact situation is similar to that of In Re Wands because the skill level is high and known administration techniques and known materials are utilized in the practice of the invention. In addition to such factual parallelism,

Appellant provided expert objective evidence in Paragraph 7 of the Fourth Supplemental Declarations of Drs. Heuser and Lorincz (of record). These medical experts read portions of the specification setting forth the generic growth factor invention and claimed and non-claimed species of such generic invention and determined that one skilled in the medical arts, armed with the guidance and direction in the relevant specification disclosures, would be enabled to practice the methods defined in the claims on appeal and to predictably anticipate the results defined therein without need for resorting to undue experimentation. When the guidance and direction provided by Appellant's specification disclosure, the level of knowledge and the content of the prior at the time of the invention, such as that of Isner, Asahara and Nabel, as established in the record and Appellant's declaration evidence are interpreted in a reasonable manner, an analysis considering the Wands factors compels a conclusion that undue experimentation would not be required to practice the invention called for in the appealed claims.

In summary, Appellant believes that the PTO failed to provide sufficient objective evidence or reasoning to support a determination of lack of enablement under current law when considered *vis-à-vis* the evidence of enablement provided by Appellant's specification. Thus, the PTO has failed to establish a *prima facie* case of lack of enablement, and this rejection should be withdrawn.

Assuming, *arguendo*, that the PTO somehow met the burden of establishing a *prima facie* case of lack of enablement, Appellant believes that any such case has been rebutted by the submission of Declarations of experts in the field – Drs. Meger, Lorincz, and Heuser. The conclusions set forth in the respective Declarations establish and rely upon objective facts that are material to a determination of enablement. Dr. Meger's

Declaration established that the disclosed administration techniques were known as of the filing date of the application. Regarding the Declarations of Drs. Lorincz and Heuser, these highly skilled medical experts read, understood, and relied upon relevant portions of the specification, including portions relating to the growth factor genus and species thereof, and based upon such objective facts, reached the determination that one skilled in the medical art, armed with the knowledge in the disclosures, would be enabled to practice the claimed method and to predictably anticipate the results defined therein without need for resorting to undue experimentation.

Appellant further points out that Drs. Heuser and Lorincz, in Paragraph 7 of their above-mentioned respective Fourth Supplemental Declarations, noted specific disclosures in the specification and stated that such disclosures related to using a growth factor for promoting the growth of soft tissue, and more specifically, to a method of using a cell, such as a stem cell, to grow soft tissue, such as an artery. Such statements rely in-part upon Appellant's disclosure at pages 40-42, which describe stem cells harvested from bone marrow, harvested from blood or from cell culture techniques, which differentiate during morphogenesis to form organs. Appellant believes that the expert opinions of Drs. Heuser and Lorincz, based upon their complete reading of the specification, fully rebut the PTO's position and confirm that of Appellant. Hence, the PTO's contention that the specification does not describe and enable a skilled medical person to grow an artery with use of a cell is erroneous.

The PTO, at page 30, ¶32 of the Rejection, states that consideration has been given to the Declarations of Dr. Heuser and Dr. Lorincz (ten declarations of record, which are set forth as Items 3-12 on the attached Evidence Appendix) but concludes that

opinions of experts in regard to the ultimate legal conclusion of enablement are entitled to no weight, citing In re Lindell and In re Chilowsky for precedent. The cited case law was purported to stand for the proposition that enablement is a question of law. However, it is clear from MPEP 2164.05 that declarations are evidence that must be considered and that weight must be accorded based on the factual evidence presented therein supporting a conclusion of enablement. The Court in In re Buchner, supra, held that "expert's opinion on the ultimate legal conclusion must be supported by something more than a conclusory statement." In Buchner, the PTO determined that the specification lacked enablement because elements necessary for carrying out the invention were neither disclosed therein nor well-known to those of ordinary skill in the art. The Court, while recognizing that the Buchner specification need not disclose what is well known in the art, agreed with the PTO that unless the identified missing elements were well-known in the art that the application must provide such information and that "it is not sufficient to provide it only through an expert's declaration." The present factual pattern is clearly distinct from that of Buchner in that the PTO has conceded that the administration of cells was known in the medical art at the time of the present invention (See page 22, first paragraph, of the September 22, 2006 Final Rejection issued in co-pending application Serial No. 09/836,750 attached hereto as Exhibit H). It is further established in this record that the compositions (stem cells, such as bone marrow stem cells), implantation apparatus (hypodermic needle) and treatment methods disclosed in the specification were well-known in the medical art. Contrary to the PTO's position, Appellant's evidence of enablement is supported by more than Declarants' conclusory statements. Declarants identify and rely upon facts, i.e., specific portions of the disclosure in the instant

specification which supports their conclusions that one skilled in the art would be able to make and use the claimed invention. Declarants' reading and understanding of the previously identified portions of the specification mentioned in Paragraph 7 of the above-mentioned Fourth Supplemental Declarations of Dr. Heuser and Dr. Lorincz, compels a conclusion that Dr. Elia was in possession the concept of implanting bone marrow stem cells and growing arteries in a human patient.

A concise reading of the multiple Declarations of Drs. Heuser and Lorincz reveals that these experts relied upon the guidance and direction in the application's generic and specific disclosures pertaining to the claims coupled with their skills and experiences in the medical art in rendering their conclusions. Appellant, likewise, relies upon such disclosure.

Other than stating at page 30, ¶32 of the Rejection that the PTO's position can be found in the record, the probative value of Appellant's evidence has not been assessed. Rather, the declaration evidence has been dismissed as "not persuasive." By failing to articulate adequate reasons to rebut the Declarations of Drs. Heuser and Lorincz, the PTO "failed to consider the totality of the record for the purpose of issuing a final rejection and thus erred as a matter of law." In re Alton, 76 F.3d 1168, 37 USPQ2d 1578 (Fed.Cir. 1996). It is trite law that the PTO must consider the probative value of such evidence vis-à-vis any asserted prima facie case. See In re Oetiker, at 1445, 24 USPQ 2d at 1444. In re Keller, 642 F.2d 413, 208 USPQ 871, (CCPA 1981). In the absence of critical analysis, the PTO appears to be relying solely upon its opinion rather than assessing weight to the objective evidence proffered in the Declarations. PTO Examiners, not being skilled persons in the medical art, must give weight to these expert opinions rather than

substitute personal opinions. See In re Neave, 370 F.2d 961, 152 USPQ 274, (CCPA 1967).

The Board's attention is respectfully directed to In re Wands, 858 F.2d 731, 737, 8 USPQ 2d 1400, 1404 (Fed. Cir.1988), which decision led to the grant of a patent. The Court found that the PTO's determination of nonenablement was unsupported by the evidence in the record. The Court further noted that the skill level in the art was high and that known materials were utilized in the practice of the invention in weighing the evidence. The instant fact situation is similar to that of In re Wands, supra because the skill level is also high and known administration techniques and known materials are also utilized in the practice of the invention. In addition to such factual parallelism, Appellant provided expert objective evidence in the form of the Declarations of Drs. Heuser and Lorincz. These medical experts read relevant portions of the specification setting forth the generic invention and elected and non-elected species of such generic invention and determined that one skilled in the medical art, armed with the guidance and direction in the specification disclosures, would be enabled to practice the methods defined in the claims on appeal and to predictably anticipate the results defined therein without need for resorting to undue experimentation. Regarding complexity, the Board is again referred to the spontaneous utterances mentioned above wherein the process was characterized as being simple by doctors skilled in the art. When the guidance and direction provided by Appellant's specification disclosure, the level of knowledge and the content of the prior at the time of the invention as established in the record, the high level of skill in the art, and Appellant's declaration evidence are interpreted in a reasonable manner, analysis

considering the factors in In re Wands compels a conclusion that undue experimentation would not be required to practice the invention called for in the appealed claims.

Once the relevant materials and administration techniques set forth in Appellant's specification and those known in the art when the application was filed, are properly considered in their entirety, Appellant believes that there should be no question that one skilled in the medical art is enabled to make and use the claimed invention. This conclusion is reinforced by the fact that the materials and administration techniques, but not the inventive result, were well known when the instant application was filed.

For the above reasons, Appellant submits that the rejection of claims 403, 411, and 412 for lack of enablement under 35 U.S.C. §112, second paragraph, is contrary to current law, and perforce, should be withdrawn.

Rejection of Claims 404 and 405 under 35 U.S.C. §112, First Paragraph Enablement

Appellant hereby repeats and relies upon the above presented remarks regarding the rejection of claims 403, 411, and 412 and submits the following remarks in support of the enablement of claims 404 and 405.

Claims 404 and 405 depend from claim 403 and call for intramuscularly injecting stem cells into a patent's leg (claim 404) or heart (claim 405). The specification, page 21, broadly describes administering soft tissue promoting compositions using a hypodermic needle. The specification, at page 45, discloses intramuscularly injecting such compositions into the leg or heart to promote the growth of an artery. Examples 18 and 19 describe specific protocols for intramuscular injection into the leg (Example 18) and the heart (Example 19). Pages 47 and 48 of the specification describe reimplanting a

patient's own cells, i.e., stem cells to promote direct differentiation and morphogenesis into an organ, such as an artery.

Those workers versed in the medical art are well aware of the techniques employed for isolating mononuclear stem cells from bone marrow and peripheral blood. The practice of intramuscular injection of therapeutic agents is so common and well known in the medical art that the PTO should take Official Notice of this fact in evaluating the of scope of enablement provided by the specification.

Rejection of Claims 407-410
under 35 U.S.C. §112, First Paragraph – Enablement

Appellant hereby repeats and relies upon the above presented remarks regarding the rejection of claims 403, 411, and 412 and submits the following remarks in support of the enablement of claims 407-410.

Claims 407-410 depend directly or indirectly from claim 403 and call for stem cells harvested from bone marrow or blood. The specification, at page 45, discloses intramuscularly injecting such compositions into the leg or heart to promote the growth of an artery. As noted earlier, the specification describes using adult (autologous) stem cells harvested from the bone marrow or peripherial blood of the patient. See pages 40-42, 47, and 48 of the specification in this regard. Pages 47 and 48 of the specification describe reimplanting a patient's own cells, i.e., stem cells to promote direct differentiation and morphogenesis into an organ, such as an artery.

Stem cells and the practice of handling, storing, culturing and implantation of stem cells, including those of the patient, harvested bone marrow and blood are so

common and well known in the medical art that the PTO should take Official Notice of these facts in evaluating the scope of enablement provided by the specification.

For the above reasons, Appellant submits that the rejection for lack of enablement under 35 U.S.C. §112, second paragraph, of claims 403-405 and 407-412 is contrary to current law, and perforce, should be withdrawn.

Provisional Rejection–Nonstatutory Obviousness-Type Double Patenting

Claims 403-405 were provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 163, and 170-173 of co-pending application Serial No. 10/179,589. Appellant again notes such rejection and stands ready to submit an appropriate Terminal Disclaimer upon an indication of allowable subject matter related to such claims.

New Provisional Rejection–Nonstatutory Obviousness-Type Double Patenting

Claims 382-406 were newly provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 161-164 and 172-174 of co-pending application Serial No. 10/179,589. It is noted that claims 382-402 and 406 of the instant application were cancelled and the new provisional obvious-type double patenting rejection is moot because it pertains to cancelled claims. As to remaining pending claims 403-405, such claims were already provisionally rejected on the ground of nonstatutory obviousness-type double patenting over claims 163 and 170-

173 of co-pending application Serial No. 10/179,589. Moreover, this rejection, to the extent that it relies upon claims 163, 172, and 173, is redundant to the prior obviousness-type double patenting rejection. Thus, the instant rejection is only new in the sense that claims 161, 162, 164, 170, 171, and 174 have been added to the prior obviousness-type double patenting rejection. The PTO's statement at page 36, ¶41 of the Rejection that, "Therefore, Applicant indicates that step (b) inherently occurs every time step (a) is performed" is not correct. Such statement is only correct when a bud is formed. In any event, Appellant stands ready to submit an appropriate Terminal Disclaimer upon an indication of allowable subject matter related to such claims.

CONCLUSION AND RELIEF SOUGHT

In view of the foregoing, Appellant urges the Board to reverse the outstanding rejections of claims 403-405 and 407-412 and respectfully requests that the instant application be passed to issue.
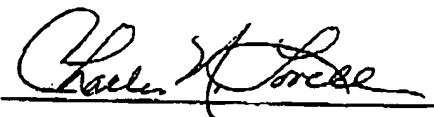
Respectfully submitted,

Dated: Aug. 17, 2009

Gerald K. White
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Attorney for Appellant

Dated: Aug. 17, 2009

Charles N. Lovell
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CLAIMS APPENDIX

Claims 403-405 and 407-412 are pending in the application, are under rejection, are being appealed, and are listed below.

LISTING OF CLAIMS

Claim 403   A method for growing and integrating tissue consisting of desired soft tissue at a selected site in a body of a human patient wherein said desired soft tissue comprises a desired artery comprising the steps of:

(a) locally injecting stem cells into said body at said selected site;

(b) forming a bud at said selected site; and

(c) growing said desired artery from said bud wherein said artery integrates itself into said body of said human patient at said selected site.

Claim 404   The method of claim 403, wherein said selected site comprises a damaged site in a leg of said patient and said stem cells are injected intramuscularly.

Claim 405   The method of claim 403, wherein said selected site comprises a damaged site in a heart of said patient and said stem cells are injected intramuscularly.

Claim 407   The method of claim 403, wherein said stem cell

comprises a living stem cell harvested from bone

marrow.

Claim 408   The method of claim 407, wherein said bone marrow is

from said patient.

Claim 409   The method of claim 403, wherein said stem cell

comprises a living stem cell harvested from blood.

Claim 410   The method of claim 409, wherein said blood is from

said patient.

Claim 411   The method of claim 403 further comprising determining

blood flow through said desired artery.

Claim 412   The method of claim 403 further comprising observing

said desired artery.

EVIDENCE APPENDIX

1. Declaration of Dr. C. Gene Wheeler cited by Appellant as an Exhibit in the Amendment filed February 15, 2001.

2. Declaration of Dr. Wayne H. Finley cited by Appellant as an Exhibit in the Amendment filed February 15, 2001.

3. Declaration of Dr. Andrew E. Lorincz cited by Appellant as an Exhibit in the Amendment filed February 15, 2001.

4. Supplemental Declaration of Dr. Andrew E. Lorincz filed November 15, 2004.

5. Second Supplemental Declaration of Dr. Andrew E. Lorincz cited by Appellant as Exhibit D in the Response filed June 26, 2006.

6. Third Supplemental Declaration of Dr. Andrew E. Lorincz cited by Appellant as Exhibit B in the Response filed April 30, 2007.

7. Fourth Supplemental Declaration of Dr. Andrew E. Lorincz cited by Appellant as Exhibit E in the Response filed November 28, 2007.

8. Declaration of Dr. Richard Heuser filed November 22, 2004.

9. Supplemental Declaration of Dr. Heuser filed June 20, 2005.

10. Second Supplemental Declaration of Dr. Heuser cited by Appellant as Exhibit C in the Response filed June 26, 2006.

11. Third Supplemental Declaration of Dr. Heuser cited by Appellant as Exhibit C in the Response filed April 30, 2007.

12. Fourth Supplemental Declaration of Dr. Heuser cited by Appellant as Exhibit D in the Response filed November 28, 2007.

13. Molecular Biology of the Cell, 4th Ed., Chapter 17, cited by the Examiner in the October 2, 2008 Office Action issued in co-pending application Serial No. 09/836,750 (also attached hereto as Exhibit A)

14. Isner U.S. Patent No. 5,980,887 cited by Appellant as Exhibit A in the Response filed November 28, 2007.

15.   Asahara, et al., 1997 _Science_ article entitled, "Isolation of Putative Progenitor Endothelial Cells for Angiogenesis" cited by Appellant as Exhibit A in Appeal Brief filed October 14, 2008.

16.   Nabel U.S. Patent No. 5,328,470 cited by Appellant as Reference AD in the Information Disclosure Statement filed February 15, 2001.

17.   Declaration of Dr. G. Robert Meger cited by Appellant as an Exhibit in the Amendment filed February 15, 2001.

18.   Final Office Action issued May 5, 2008, page 14, paragraph 18 (also attached hereto as Exhibit B).

19.   The Journal of Invasive Cardiology, Vol. 17, July 1, 2005, entitled "Progenitor Cell Transplantation and Function following Myocardial Infarction" (author unknown) cited by the Examiner in the October 2, 2008 Office Action issued in co-pending application Serial No. 09/836,750 (also attached hereto as Exhibit C—complete copy).

20.   The Journal of Invasive Cardiology, Vol. 17, July 1, 2005, entitled "Progenitor Cell Transplantation and Function following Myocardial Infarction" (author unknown) cited by the Examiner in the October 2, 2008 Office Action issued in co-pending application Serial No. 09/836,750 (also attached hereto as Exhibit C1—incomplete copy).

21.   The Journal of Invasive Cardiology, Vol. 17, July 1, 2005, entitled, "Tissue Engineering and Interventional Cardiology" cited by the Examiner in the October 2, 2008 Office Action issued in co-pending application Serial No. 09/836,750 (also attached hereto as Exhibit D-complete copy).

22.   The Journal of Invasive Cardiology, Vol. 17, July 1, 2005, entitled, "Tissue Engineering and Interventional Cardiology" cited by the Examiner in the October 2, 2008 Office Action issued in co-pending application Serial No. 09/836,750 (also attached hereto as Exhibit D1-incomplete copy).

23.   Murry et al. 1996 publication in J. Clin. Invest. entitled, "Skeletal Myoblast Transplantation for Repair of Myocardial Necrosis" cited by the Examiner in the November 28, 2003 Office Action issued in co-pending application Serial No. 09/836,750 (also attached hereto as Exhibit E)

24.   Caplan et al. publication in Journal of Orthopaedic Research, entitled, "Mesenchymal Stem Cells" cited by Appellant as Reference ACA in the Sixth Supplemental Information Disclosure filed February 21, 2006.

25. Strauer et al. 2002 publication in <u>Circulation</u> entitled, "Repair of Infarcted Myocardium by Autologous Intracoronary Mononuclear Bone Marrow Cell Transplantation in Humans" cited by Appellant as Reference ABQ in the Third Supplemental Information Disclosure Statement filed May 27, 2003.

26. Kornowski U.S. Patent No. 7,097,832 cited by Appellant as Exhibit B in the Response filed November 28, 2007.

27. Third Supplemental Declaration of Dr. Heuser (originally filed in co-pending application Serial No. 10/179,589) and cited by Appellant as Exhibit B in the Letter filed May 25, 2007.

28. Second Supplemental Declaration of Dr. Andrew E. Lorincz (originally filed in co-pending application Serial No. 09/794,456) and cited by Appellant as Exhibit A in the Letter filed May 25, 2007.

29. Dohmann, et al., 2005 publication in <u>Circulation</u>, entitled, "Transendocardial, Autologous Bone Marrow Mononuclear Cell Injection in Ischemic Heart Failure" cited in Examiner's Answer, dated November 28, 2007, in co-pending application Serial No. 09/794,456 (also attached hereto as Exhibit F)

30. Strauer et al. 2005 publication in <u>Circulation</u> entitled, "Regeneration of Human Infarcted Heart Muscle by Intracoronary Autologous Bone Marrow Cell Transplantation in Chronic Coronary Artery Disease" cited by Appellant as Exhibit D in an Amendment dated November 21, 2005 in co-pending application Serial No. 09/974,456 (also attached hereto as Exhibit G)

31. Final Office Action dated September 22, 2006, page 22, first paragraph, issued in co-pending application Serial No. 09/836,750 (also attached hereto as Exhibit H).

RELATED APPEALS AND INTERFERENCES APPENDIX

1. Co-pending application Serial No. 09/794,456, in which Appellant's Appeal Brief was filed at the PTO on February 6, 2009;

2. Co-pending application Serial No. 09/836,750, in which Appellant's Appeal Brief was filed at the PTO on May 27, 2009; and

3. Co-pending application Serial No. 10/179,589, in which Appellant's Appeal Brief was filed at the PTO on July 2, 2009.

Document Code: IMIS

Notice of Fee Due

Date: _08-21-09_

Application Number: _09064000_

A fee is due for the attached document for the reason indicated below. Please check the application for the appropriate authorization to charge a deposit account. If an authorization is present, please charge the appropriate fee*. If an authorization is not present, notify the application of the fee deficiency.

*If the fee due is for any of the filing fees, check for authorization to charge the surcharge. If authorization is present, charge the surcharge for late payment of the filing fees as well.

☑ Insufficient payment by check or money order.

❑ Insufficient funds in deposit account_____ at _____:_____ (time).

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Fee code(s) to be applied: _2257_   _$65.00_

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Amount in holding fee code: _1506_   _____

_1622/2622_   _____

_1999_   _$60.00_

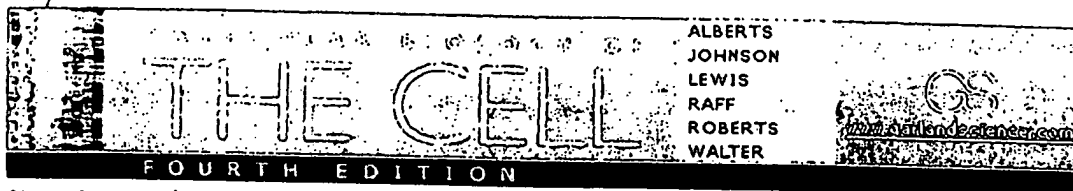Total remaining due from applicant: _$5.00_

RAM Operator _C C_

Rev. 12/27/07

EXHIBIT A

<u>Molecular Biology of the Cell</u>, 4th Ed., Chapter 17,
cited by the Examiner in the October 2, 2008 Office Action
issued in co-pending application Serial No. 09/836,750

ALBERTS
JOHNSON
LEWIS
RAFF
ROBERTS
WALTER

THE CELL

FOURTH EDITION

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Molecular Biology of the Cell

Bruce Alberts
Alexander Johnson
Julian Lewis
Martin Raff
Keith Roberts
Peter Walter

Molecular Biology of the Cell is the classic in-depth text reference in cell biology. By extracting fundamental concepts and meaning from this enormous and ever-growing field, the authors tell the story of cell biology, and create a coherent framework through which non-expert readers may approach the subject. Written in clear and concise language, and illustrated with original drawings, the book is enjoyable to read, and provides a sense of the excitement of modern biology. Molecular Biology of the Cell not only sets forth the current understanding of cell biology (updated as of Fall 2001), but also explores the intriguing implications and possibilities of that which remains unknown.

The hallmark features of previous editions continue in the Fourth Edition. The book is designed with a clean and open, single-column layout. The art program maintains a completely consistent format and style, and includes over 1,600 photographs, electron micrographs, and original drawings by the authors. Clear and concise concept headings introduce each section. Every chapter contains extensive references. Most important, every chapter had been subjected to a rigorous, collaborative revision process where, in addition to incorporating comments from expert reviewers, each co-author reads and reviews the other authors' prose. The result is a truly integrated work with a single authorial voice.

Bruce Alberts received his Ph.D. from Harvard University and is President of the National Academy of Sciences and Professor of

Biochemistry and Biophysics at the University of California, San Francisco. **Alexander Johnson** received his Ph.D. from Harvard University and is a Professor of Microbiology and Immunology at the University of California, San Francisco. **Julian Lewis** received his D.Phil. from the University of Oxford and is a Principal Scientist at the Imperial Cancer Research Fund, London. **Martin Raff** received his M.D. from McGill University and is at the Medical Research Council Laboratory for Molecular Cell Biology and Cell Biology Unit and in the Biology Department at University College London. **Keith Roberts** received his Ph.D. from the University of Cambridge and is Associate Research Director at the John Innes Centre, Norwich. **Peter Walter** received his Ph.D. from The Rockefeller University in New York and is Professor and Chairman of the Department of Biochemistry and Biophysics at the University of California, San Francisco, and an Investigator of the Howard Hughes Medical Institute.

Cell Biology Interactive
Artistic and Scientific Direction: Peter Walter
Narrated by: Julie Theriot
Production, Design, and Development: Mike Morales

Front cover Human Genome: Reprinted by permission from *Nature*, International Human Genome Sequencing Consortium, 409:860-921, 2001 © Macmillan Magazines Ltd. Adapted from an image by Francis Collins, NHGRI; Jim Kent, UCSC; Ewan Birney, EBI; and Darryl Leja, NHGRI; showing a portion of Chromosome 1 from the initial sequencing of the human genome.

Back cover In 1967, the British artist Peter Blake created a design classic. Nearly 35 years later Nigel Orme (illustrator), Richard Denyer (photographer), and the authors have together produced an affectionate tribute to Mr Blake's image. With its gallery of icons and

influences, its assembly created almost as much complexity, intrigue and mystery as the original. *Drosophila, Arabidopsis*, Dolly and the assembled company tempt you to dip inside where, as in the original, "a splendid time is guaranteed for all." (Gunter Blobel, courtesy of The Rockefeller University; Marie Curie, Keystone Press Agency Inc; Darwin bust, by permission of the President and Council of the Royal Society; Rosalind Franklin, courtesy of Cold Spring Harbor Laboratory Archives; Dorothy Hodgkin, © The Nobel Foundation, 1964; James Joyce, etching by Peter Blake; Robert Johnson, photo booth self-portrait early 1930s, © 1986 Delta Haze Corporation all rights reserved, used by permission; Albert L. Lehninger, (unidentified photographer) courtesy of The Alan Mason Chesney Medical Archives of The Johns Hopkins Medical Institutions; Linus Pauling, from Ava Helen and Linus Pauling Papers, Special Collections, Oregon State University; Nicholas Poussin, courtesy of ArtToday.com; Barbara McClintock, © David Micklos, 1983; Andrei Sakharov, courtesy of Elena Bonner; Frederick Sanger, © The Nobel Foundation, 1958.)

EXHIBIT B

May 5, 2008 Final Office Action page 14, paragraph 18

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/064,000	04/21/1998	JAMES P. ELIA	796-P-12	5311

7590   05/05/2008

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EXAMINER
GAMETT, DANIEL C

ART UNIT	PAPER NUMBER
1647	

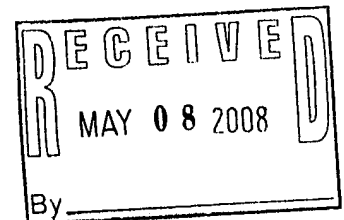| MAIL DATE	DELIVERY MODE
05/05/2008	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

PTOL-90A (Rev. 04/07)

	Application No.	Applicant(s)
Office Action Summary	09/064,000	ELIA, JAMES P.
	Examiner	Art Unit	
	DANIEL C. GAMETT	1647	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

> A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE <u>3</u> MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.
> - Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
> - If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
> - Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
>   Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on <u>28 November 2007</u>.

2a)☒ This action is **FINAL**.   2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) <u>403-405 and 407-412</u> is/are pending in the application.

   4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) <u>403-405 and 407-412</u> is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

   Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

   Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

   a)☐ All   b)☐ Some *   c)☐ None of:

   1.☐ Certified copies of the priority documents have been received.

   2.☐ Certified copies of the priority documents have been received in Application No. _____.

   3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

   * See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1)☐ Notice of References Cited (PTO-892)	4)☐ Interview Summary (PTO-413)
2)☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)	Paper No(s)/Mail Date. _____.
3)☐ Information Disclosure Statement(s) (PTO/SB/08)	5)☐ Notice of Informal Patent Application
Paper No(s)/Mail Date _____.	6)☐ Other: _____.

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PTOL-326 (Rev. 08-06)   **Office Action Summary**   Part of Paper No./Mail Date 20080227

DETAILED ACTION

1. The amendments of 11/28/2007 have been entered in full. Claims 1-402 and 406 are cancelled.

   The newly cancelled claims include claims which were rejected in previous office actions. All

   prior objection/rejections directed to cancelled claims are moot and hereby withdrawn.

2. Claims 403-405 and 407-412 are under examination.

Claim Rejections - 35 USC § 112

3. The following is a quotation of the second paragraph of 35 U.S.C. 112:

   > The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the
   > subject matter which the applicant regards as his invention.

4. Rejection Claims 403-405 under 35 U.S.C. 112, second paragraph, as being indefinite for

   failing to particularly point out and distinctly claim the subject matter which applicant

   regards as the invention is maintained and hereby extended to new claims 407-412 as they

   depend from claim 403. Applicant's arguments filed 11/28/2007 have been fully considered

   but they are not persuasive. The rejection of record held that the recitation in independent

   claim 403, step (b) "forming a bud" creates a lack of clarity as to whether the recited step

   requires action on the part of the practitioner of the method to form a bud. Applicant argues

   (p.7) that, "it is clear from the specification that the only step required by the practitioner is

   that of injecting stem cells into a selected site in a patient's body." Thus, Applicant

   acknowledges that although step (b) (and, by implication, step (c)) has the form of a method

   step, the actual intent is to recite an intended outcome. The claim defines the invention.

   Claim 403, which has not been amended, still appears to recite a method step instructing the

a nearly identical scope to the instant claims. Like the instant claims, the corresponding

claims in the '589 application have been rejected under 35 U.S.C. 112, first paragraph, but in

the rejection in the '589 application have been deemed to be enabling for a scope that

includes a method of growing and integrating a desired artery at a selected site in a body of a

human patient comprising the steps of locally placing a CD34+ mononuclear cell harvested

from bone marrow or peripheral blood in a body of a human patient. Applicant suggests that

a similar determination of enablement would be in order for the scope of subject matter set

forth in the present claims, in view of the aforementioned double patenting rejection and the

commonality of the disclosures. This is not persuasive for the following reasons.

18. Neither the instant disclosure nor '589 application teaches one of skill in the art how to

perform the claimed method of growing an artery using stem cells. However, *the state of the

art* is a factor that must be taken into consideration in determining enablement. Claims filed

in 2002 reciting methods to grow an artery using stem cells cannot be said to totally lack

enablement because, by then, the state of the art had changed so that such a method was

known to be possible. This change in the state of the art is evidenced by US Patents 5980887

and 7097832, which were cited as anticipatory disclosures under 35 U.S.C. 102(e) in the

office action mailed on 08/31/2007 in the '589 application. Thus, the rejection in the '589

application stated that the scope of enablement is supplied solely by that which is known in

the art, based upon disclosures that occurred after the filing of application 09/064,000, in

order to make it clear that, *by themselves*, neither the instant disclosure nor the '589

disclosure would support any scope of enablement. Thus the instant claims can be rejected as

lacking enablement, whereas similar claims in the '589 application are afforded a scope of

enablement supported by the state of the art. Neither of Applicant's disclosures could

possibly have guided or contributed to others' success in developing the method.

19. Next, Applicant (p. 14) suggests that the specification (pages 20, 21, 30-32, and 38-42)

provides a substantial body of disclosure regarding using a growth factor to form a bud and

grow soft tissue in a human body and that pages 10, 20, 21, 31, 32, and 37-52 describe "a

class of growth factors that broadly and specifically includes genes, nucleic acids, a patient's

own cells (autologous cells), or universal cells, e.g., stem cells (global mononuclear bone

marrow cells), etc., all of which are described to promote tissue growth through

differentiation and morphogenesis." Applicant complains that the Examiner has only

considered the disclosure regarding enablement as it specifically relates to the elected growth

factor species, cells, "which ignores Applicant's broad and specific disclosure relating to non-

elected growth factor species disclosure". This is not persuasive for several reasons. First and

foremost is the fact that the instant claims are specifically drawn to using stem cells to grow

an artery. It is altogether proper for the examination to focus on the teachings of the

specification that are directed to the claimed methods. Secondly, Applicant's argument on

page 14 contradicts the argument on page 18, wherein Applicant complained that when

teachings on pages 20, 32, 46, 47, 47, and 50 of the specification were addressed in

paragraph 32 of the previous office action, the "paragraph is gratuitously concerned with

non-elected inventions and thus lacks focus upon the claimed invention".

20. While the lexicon of this specification permits the examination of claims reciting

administration·of cells after Applicant had elected the species "living organisms", it is a

separate question whether broad disclosures such as those on pages 10, 20, 21, 30-32, and

EXHIBIT C

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Progenitor Cell Transplantation and Function following Myocardial Infarction

VOLUME: 17 PUBLICATION DATE: Jul 01 2005

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William O'Neill: I have a couple of questions for both of you. First, we are essentially talking about adult, autologous stem cells. As you all know, there is enormous controversy in the U.S. about the use of embryonic stem cells which we are currently prohibited from using. From a dispassionate, basic scientific approach, do you think there's an advantage to using embryonic stem cells, or will adult stem cells be sufficient in terms of their ability to replicate and cause myogenesis?

Peter Gonschior: After examining the data from experiences in other fields with vascular cells and their biological functions, I don't think there is a need for embryonic stem cells. The bone marrow cells appear to be very robust; even the non-randomized European trial data show that with a not-so-profound approach, it is possible to obtain positive effects. Due to the political scenery in Europe, I don't think it would be possible to conduct large clinical trials using embryonic stem cells.

Sigrid Nikol: I think we should first try to optimize the strategies using autologous cells due to the ethical concerns. Due to these restrictions, there is not currently much proof available that embryonic stem cell therapy is that much better or even less risky. We know that these cells can differentiate, which means that they could differentiate into undesired cells as well. Thus, given the legal restrictions at present, we should stick with what we can do and try to make the best of it.

William O'Neill: Another key issue that arises from a clinical standpoint is that of direct injection versus intra-arterial or intravenous infusion. Do we know yet which would be the most efficient way — if the appropriate cell were to be found — to deliver the cells for the most efficient myogenesis?

Peter Gonschior: Sigrid and I have a lot of experience with local drug delivery. Efficiency of local drug delivery is in fact very low. It would be very smart to just inject the cells intravenously. But one critical issue with all of these data is that delivery efficiency, whether systemic or local, is very low. In fact, it turned out that endocardial delivery of these cells was the most efficient, resulting in the largest number of injected cells — up to about 30 million.

William O'Neill: The problem I have is that the stem cells are very sensitive to hypoxia, they need an oxygenated environment in order to thrive. Thus, if these cells are injected into a core infarct area, it will likely be hypoperfused and the ambient oxygen tension in that area may not be sufficient to support those cells. Sigrid, your experience involved a permanent ligation, right?

Sigrid Nikol: Actually, there were two sets of animals, but I only showed you those with the permanent ligation because there are currently more data on them. We also have a reperfusion model from 30 minutes after opening up the vessel.

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Sigrid Nikol: We perform stem cell therapy via bone marrow stimulation (not cell injection). At the moment, we do not have sufficient data on the reperfusion model. The problem is that when genes or cells are injected into the vessels, why should they pass the endothelium and migrate into the tissue where they are needed? It is very unlikely to happen, unless there is injured tissue. And with the venous application, there are the first-pass effects in the lungs and liver on top of it. These findings are not very convincing in my opinion, including the data from the Strauer group which lacked a truly randomized control group. The problem with intramyocardial injections into infarcted areas is a real one, as you stated, in that there is no blood/oxygen supply and potentially arrhythmogenic foci are created. Also, there may not be a homogeneous distribution of cells — an issue that has already been discussed with regard to gene therapy for the myocardium. Specifically, the question had been raised regarding how homogeneous gene therapy in the myocardium can be achieved without creating arrhythmias. With stem cells, this may be an even more important issue, as has been demonstrated in the work by Menasche et al.

William O'Neill: It seems to me that a highway is needed to get the cells to the tissue, and that's why we have been considering autoinfusion or direct intracoronary infusion of the agents, because in the reperfusion model, at least if there's an intact vascular structure, then the environment will be ambient and oxygenated until the cells can engraft. But my question is: Why would those cells stay at that target site? Also, we are lacking basic scientific understanding of the signals that allow the stem cells to hone in on that particular tissue. Since stem cells could potentially be coming in from the systemic circulation, we need to come up with a honing material that could be injected into the infarcted area and would allow the stem cells to accumulate there. We are moving forward in this field.

At the 2003 AHA meeting, data from the late-breaking BOOST trial were presented. It was, I think, a very well-conducted randomized trial involving 60 patients with placebo versus bone marrow infusion arms. The results showed a statistically significant increase in ejection fractions in the active treatment group compared with the placebo group. TOPCARE was a terrific study, but it was a sort of historical control study. Thus, we are beginning to accumulate data that suggest there is some merit to this approach. Let me turn this question over now to the other panelists. Matt Pollman, from Guidant Corporation, is on the panel. Matt, is there business potential in this field?

Matt Pollman: That's a great question, Bill. As a clinician and a scientist, this area is extremely

provocative and intriguing, particularly the data from the BOOST and TOPCARE myocardial infarction trials. There is an aggressive movement to push these trials out into more randomized, controlled, multi-center arenas. The issue for a company such as Guidant is what we can bring to the table and whether there is a business potential in this area. Confidentiality, of course, prevents me from saying too much here, but there certainly appear to be business opportunities for companies like ours. We believe that it will require a very safe delivery system that can be applied to a wide range of patients. The cells clearly need to be delivered at least very close to the target site in order to successfully do their job. In that light, an intracoronary infusion strategy raises questions about how to allow enough time for the cells to adhere, to transmigrate, enter the tissue and do their job. There is room for improvement in the delivery systems, and that's what Guidant is looking into. The TOPCARE acute myocardial infarction study headed up by Andreas Zeiher in Frankfurt uses a simple syringe injection system loaded with 10 cc of bone marrow, 3 cc of which is applied to the coronary arteries. The patients are then allowed to be in an unreperfused state for 3 minutes to allow the cells to do their job. Often, the limiting factor is that the patients complain of chest pain during the unreperfused state. Thus, there are opportunities to improve the current infusion protocol.

William O'Neill: We have been looking at it in a two-prong fashion, with a lot of interest in basic science on one end, and in clinical trials on the other end. As for the United States, if bone marrow is taken from a patient and then reinjected, the FDA considers it non-homologous use, even though it is not a drug or a commercial compound. The U.S. FDA regulations on stem cell use do not cover autologous use of these cells. Unfortunately, thus, this entire field has become embroiled in the embryonic stem cell and abortion debates. As a result, the FDA is incredibly cautious about allowing embryonic stem cell use. The FDA regulations state that if the stem cells are taken and not manipulated, and then reinjected, it falls outside of the FDA's authority. At the 2003 TCT meeting, the FDA representative specifically stated that if any compound is taken from the body and reinjected (i.e., spinal fluid) into an area where it doesn't normally need to be, then the FDA does have regulatory authority.

Also, the FDA insists on receiving a significant amount of data — and rightfully so — on basic safety issues such as clotability and other matters surrounding the infusion of these cells into the coronary arteries. I think it will be another year or two in the U.S. before the basic science data are available to allow clinical trials to proceed. The bulk of the basic scientific studies will be carried out in South America and Europe.

Having said that, we have a perfect opportunity right here to learn from our colleagues about where this field is headed internationally. I know that you, Alfredo, are very much in the midst of all of this research. Would you mind telling us what your group will be doing in terms of your randomized trial?

Alfredo Rodriguez: Thank you, Bill. We are just starting a randomized trial that will follow the rules of the TOPCARE MI trial. Our trial involves 40 patients, 20 in each arm. One patient arm receives autologous bone marrow injections. These are acute myocardial infarction patients from 3 to 12 hours after symptom onset; all patients receive percutaneous coronary intervention and stenting. After reperfusion, we randomize the patients on day-4. On day-5, we puncture the patient's iliac crest, and the next day, we infuse the drug in 10 ml of solution into the coronary arteries.

The patient undergoes angiography immediately following the PCA procedure. Global and regional ejection fractions are measured. An acute and 4-month dobutamine stress echocardiogram is then done, followed by an MRI and SPECT imaging.

Our institution has a very active bone marrow transplant team. The hematologists who serve on our trial's executive committee told us that it was not necessary to place this trial under the Argentinian equivalent of the FDA, because autologous bone marrow is not a drug; it's not foreign material to the body. Thus, our trial is approved by the local transplant agency. My concern involves legal problems that could arise. I would like to hear from Sigrid and the other European colleagues here if the ongoing clinical trials in Europe in this field are approved by their respective regulatory agencies, or if they are only approved as protocols by the local hospitals' scientific committees, with the patients of course providing informed, written consent.

Sigrid Nikol: According to the blood transfusion and federal drug laws, there are certain regulatory approvals needed, particularly if the doctor obtaining the cells is not the same doctor using them therapeutically. In this case, cells are considered a blood product and their use is regulated.

Alfredo Rodriguez: I do know that the TOPCARE trial did not have local German regulatory approval.

Richard Heuser: I assume that we're talking here about a normal 10 cc bone marrow aspirant — no filtering — just administering it down the coronary arteries. Is that correct? And then the balloon is inflated for 3 minutes or so to allow the cells to disperse? And several injections are given?

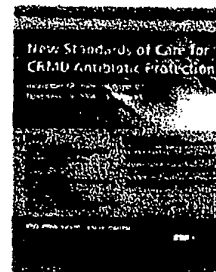**Alfredo Rodriguez:** Yes, 3 or 4 injections are given.

William O'Neill: This approach has generated controversy due to the fact that the bone marrow is unfiltered and thus contains fat, spicules, mesenchymal cells, and so on. Basically, the injection contains the "kitchen sink" and we hope that the right cells go to the right place and do the right thing. The other argument is that we know which cells we want, so we should just take them, filter them, grow them in media and replicate them, increase their efficiency, and then inject them. Those are the two schools of thought on the subject, but I can't tell you which is the correct one, because we might not have the right cells. It may be that the CD34 positive cells are not the right ones. In the TOPCARE study, they actually look both the peripheral cells and the cells obtained through leukophoresis, then identified them, segregated them, and grew them in a culture medium to increase their numbers.

In terms of FDA regulations, whenever you manipulate and produce cells, a commercial product results, and thus clearly falls under the FDA's purview. A regulatory "gray" area still exists in the U.S. when it comes to simply taking cells, leukophoresing them, removing the stem cells, and reinfusing them.

Richard Heuser: The first time I saw this technique presented by the group in Frankfurt, I was astonished at how simple it actually was. I am surprised that I didn't get into regulatory trouble myself about 5 or 6 years ago when I treated a patient in the middle of the night who tore a coronary artery. At that time, I had our home-made covered stents and some JoMed stents, but the vessel was 2.3 mm, and the patient was in cardiogenic shock. I had administered ReoPro and t-PA to this 70-year-old female patient. I just took some clot, combined it with a little of the protamine, and it got to be enough of a slurry. I then put it down with a balloon, occluded the vessel, then re-opened it — and it was sealed. I then stented the vessel, and it was fine. But I find it hard to believe that if we administer these bone marrow cells to a patient with a huge infarct that we could get into trouble with the FDA. Some of these therapies make good sense for the individual patient, but more study data are needed.

William O'Neill: Let me pose a question to Paul Overlie, who has had extensive experience treating acute myocardial infarction patients for the past 20 years. Is there a need for this, Paul? The CADILLAC and recent myocardial infarction studies showed a 2% mortality rate and an ejection fraction mean of about 50%. How often does the situation arise that would warrant going to the trouble of doing bone marrow aspirates and leukophoresis on these patients?

Paul Overlie: The very high-risk, no-reflow patients might benefit from these therapies. Once these bone

marrow cells are aspirated, is there some way to get the cytokines activated before injecting them, or concentrating them, so that the U.S. FDA would approve of the technique?

William O'Neill: Now that more studies have been conducted since the last time — about a year-and-a-half ago — that the FDA was approached about this, it's likely that the FDA will show more interest in allowing U.S. clinical trials to go forward. Phil, do you think there's an application for the genetic or stem cell repair approaches in the general vasculature — the aorta or peripheral vasculature?

Philip Walker: I am a peripheral vascular surgeon, so I am definitely interested in myocardial repair to get our patients fit for intervention or following infarcts after intervention. There are a number of emerging areas where the approach might be helpful. Peter mentioned the single-center study on stem cell use for peripheral revascularization which involves an area where patients are nonreconstructible, particularly diabetics with renal failure.

I also wonder whether the no-reflow phenomenon — perhaps even in the setting of acute limb ischemia — might benefit from stem cell therapy. Stem cells may also be useful as an adjunct to tissue engineering. I work with a group who are developing a biological graft based on a peritoneal growth, which may be another useful area for the adjunctive use of stem cells. This therapy is being developed with the aim of improving the antithrombotic effects, which might also apply to prosthetic grafts that have been plagued by thrombotic problems when small diameters are involved.

Aortic repair in patients who have not yet developed sizable aneurysmal disease may be yet another area for stem cell therapy, but we need to learn how to identify these patients. We also need to learn to identify patients with small aneurysms, as stem cell repair might be useful in repairing and inhibiting the process in these patients.

Another viable area may be in the area of stroke and revascularization, as well as brain repair. This raises the issue of whether the mechanisms will be generic across all of those vascular beds, or whether differences exist, and whether the basic science needs to be worked out for the different areas.

I would like to ask about the issue of toxicity, particularly in diabetic patients in whom there may be an acceleration of diabetic retinopathy, tumorigenesis in the elderly patients, as well as plaque instability. Are these issues relevant?

William O'Neill: Perhaps because these bone marrow cells are pluripotential and have stimuli for differentiation, they will probably not be carcinogenic. And since they serve repair processes, it is unlikely that they will cause pathologic proliferation. Those are all critical questions that have plagued the field of gene therapy in which vectors were found that caused some cells to proliferate wildly. I think these cells will be safer, but we really won't know until a large number of patients are treated.

From our own acute myocardial infarction work, I presented a slide on the number of patients who present within 2 hours of symptom onset, and that number is about 5% of the U.S. acute myocardial infarction population — at least with the current standards. Perhaps with more novel, patient-directed approaches, this percentage could rise. After 2 hours, whether the patient is reperfused or not, there will be a substantial amount of necrotic tissue and a large permanent infarct zone. If stem cell therapy could be safely applied, I believe that many patients could benefit in terms of improved regional function, making an akinetic anterior wall hypokinetic, or improving or preventing aneurysm formation.

Brian O'Murchu: Has the coronary sinus retrograde perfusion route been used for administration of these cells?

William O'Neill: Not that I am aware. There is one company that makes a device for access to the coronary sinus, and then needle injection into the myocardium. I think there may be some interest in using that as an access site rather than performing ventricular puncture.

Brian O'Murchu: I was just talking with my colleague, Alex Zapolanski, about whether the solution emerges from the ostia of the arterial coronaries during retrograde coronary perfusion, and of course it does. Thus, it would seem to provide the opportunity to "bathe" the myocardium through the use of a system that can be balloon-occluded, allowing perfusion to be maintained.

William O'Neill: That has been discussed, but I am not aware of any ongoing trials on that topic.

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EXHIBIT C1

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Progenitor Cell Transplantation and Function following Myocardial Infarction

VOLUME: 17 PUBLICATION DATE: Jul 01 2005

Issue Number:
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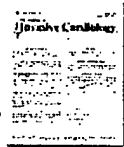
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Tissue Engineering and Interventional Cardiology

VOLUME: 17 PUBLICATION DATE: Jul 01 2005

Issue Number:
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author:

Speaker: David Holmes, MD
Moderator: Reginald Low, MD
Panel members: George Dangas, MD, Wolfgang Ritter, MD, Peter Gonschior, MD, Brian Firth (Cordis Corporation)

George Dangas: There have been many attempts and many failures in this field due perhaps to the inherent tendency of interventional cardiologists to move quickly and work from assumptions, applying what may not yet be well understood. Gene therapy and engineered viruses (associated viruses, attenuated viruses, etc.) are examples of this. When it became clear that we were unable to identify the most appropriate and effective agent for angiogenesis, we looked toward the newly fashionable stem cell-based therapies. Even researchers make 3 million agents, and 2 of them turn out to be effective, that would be fine. On the other hand, perhaps the stem cells will produce 2 or 3 agents that work for angiogenesis, but at the same time, 1 or 2 other agents produce negative effects — the result being that the positive effect hoped for is not achieved.

Thus, the interventional cardiology field must achieve more "crisp" results based on more "crisp" basic science, with better-established findings, in order to better understand what the targets are and pursue them in a more methodological manner. Our methodology needs to be evidence-based, as opposed to the focusing on the practicalities of how to achieve our aims. We need to scale down the in vivo applications and return to the laboratory.

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shown that when these different sorts of cells are delivered intravenously, they go to the lungs and have a "tremendous time," and they don't reach the myocardium. So while it makes perfect sense to use the intravenous approach, these cells are filtered out in the lungs and remain there. If those cells are active and produce cytokines, perhaps that's all we would want to use them for. Maybe these cells aren't the magic solution, and maybe we don't have a clue about this. Perhaps we can use these cells for the cytokines they produce systemically and they will cause other bone marrow cells to home in on the site of injury. But at the present, we just don't know enough about this process.

Patrick Whitlow: I just want to give you an update on Bioheart because of their underlying disease process, these patients are very prone to arrhythmia and sudden death. And theoretically, if you are adding islands of tissue in the left ventricle that is already damaged, these islands of tissue are not enervated in the same way as the surrounding tissue and the conduction properties aren't the same. You would theorize that this could set up re-entry circuits. Thus, ventricular arrhythmia presents an enormous problem in terms of conducting studies because many of these patients are going to die from their underlying disease. To detect if cell injection causes worsened arrhythmias will be very difficult, but a potentially serious problem. Therefore, the first v.s. clinical trial involves patients who already have defibrillators, and the number of patients will be small because of the need for defibrillators. The study should answer the question of whether this is arrhythmogenic — which Patrick Serruys believes is the case. Other researchers in France don't believe that injecting cells is arrhythmogenic. Who knows? It will take a long time and a lot of patients to arrive at the answer.

If a start-up company tries to make this therapy work, it will be very difficult for industry to actually fund the research from start to finish. We know from the animal studies that efficacy increases with higher doses of cell therapy, but we have yet to find what a potentially toxic dose is for the size of the island of cells that produce arrhythmias. I think that the bulk of this research will have to be federally funded. It's all very interesting work, and according to the animal models, it should work in humans.

David Holmes: Although the skeletal myoblasts appear to be arrhythmogenic, it appears to relate to engraftment properties. With true stem cells — whatever they are — it doesn't seem to be as problematic, whether because there have so far been very small numbers of patients, or whether indeed these stem cells are more pluripotential and engraft better, or whether they are more homogeneously distributed, and aren't just islands. It is early in this field, and I would echo what George said: in a field where so much rides on a product or technique, some of the trials are too premature because we often don't have the necessary solid scientific underpinnings before launching an important large trial. The biggest potential problem downstream to this approach is that if the product fails, we don't know for certain whether failure was due to the product's ineffectiveness or because we didn't know how to properly use it.

George Dangas: I would like to comment on interpreting the data from some of these early studies. I don't think we have the proper tools to accurately study the early results. The preliminary decision by the Rotterdam group was to implant defibrillators in all patients of the Bioheart study after two or three deaths occurred in one arm. Still, we haven't figured out whether it was actually the number of implants or if it was a patient substrate with a number of implants that caused the arrhythmogenecity. I think that any other study at this stage would produce statistical errors in both directions, which makes it very difficult to determine whether it was a failure of the agent, the liver system, or that the patients in the treatment arm were too sick and were going to die anyhow. That last explanation is a possibility because, due to ethical considerations, we usually enroll "no option" patients for these types of agents.

Richard Heuser: The Bioheart study involves a specific, patentable therapy which provides greater incentive to the company to see the project through to the end. One thing that always concerns me is determining what the endpoint will be. We all love to see these ejection fractions, but I think that the two main endpoints will likely be treadmill time (endurance) and objective findings of symptom relief. A third endpoint might be the number of hospitalizations for congestive heart failure. I agree that we have to conduct this study in some sort of randomized fashion. I think that the low-dose cells which we discussed will be a good way to do it. Also, since it's a very small number of patients being subjected to this very expensive therapy, I wonder if we could collect data on the patients before we commence therapy. In other words, we would assign the patient. We all know how long it takes to enroll patients in this trial; there's a lot of information to gather. During the six-month lead-in period, more data points could be obtained by looking at retrospective data on those individual patients. It won't be enough to see the ejection fraction increase, and there certainly won't be a reduction in mortality.

David Holmes: I think there will be a reduction in mortality rates and it will be the lead-in phase. For instance, all of the transplant centers have patient deaths while on the waiting list. This study will provide the same type of information. There may be other endpoints — viability of MR, for example. Whether viability with MR will be an "approvable" endpoint remains to be seen, however. We will need to be creative in terms of endpoints.

William O'Neill: I agree with you, George, in terms of the degree of our ignorance about the basic science in this area. My own feeling is that God — or nature — in His infinite wisdom, is a lot smarter that we will be for a few centuries yet in terms of the cascade of processes that actually allow a new cell to come in and regenerate. It is a little foolhardy to say that we should wait until we completely understand these processes before any clinical trials are launched. These early attempts are fine, as long as patients aren't harmed and as long as the patients are properly selected in terms of their ability to spontaneously improve function. As you said, pre-transplant patients will not improve function and there will likely be a big upside and very little downside for them. I would thus encourage conducting these small, mechanistic trials as a means of enlightening us as to where we stand and where we must go. Finally, when we change from the basic experiments to human trials, we are dealing with patients who are on all types of medications. Do ACE inhibitors, calcium channel blockers, beta-blockers and nitrates alter, improve or decrease the ability of cells to regenerate? We simply don't know the answer to this question. I do believe that we face a long process of trial and error, and will make small advances along the way.

David Holmes: I think that view is correct, provided that if the small trials are negative, we don't then abandon the field and decide that the therapy doesn't work. It seemed to be the case with some of the gene therapy trials where incredible hype was followed by randomized trials that produced negative results, setting the field back significantly. I think that well-designed studies aimed at identifying mechanisms will be terribly important for the field.

Brian Firth: In terms of endpoints, I believe that this falls under the same rules as most of the heart failure studies. The FDA wants to see that therapies designed for patients with heart failure or impending heart failure don't increase mortality while improving other parameters. Thus, researchers don't have to prove that the therapy improves survival rates, but they do have to prove that it doesn't adversely affect survival. That was the big lesson learned from the inotropic therapy studies.

Thomas McNamara: What has been the progress and/or expectations with other critical organs — namely, the liver and the kidneys? Has work been done in this area?

David Holmes: I think work has been done, particularly on the liver, partly because it can regenerate. We tend to think that heart cells will repair what has been a problem, and I don't know if they will wildly proliferate and make a totally new heart, liver cells can do. You need to understand that I'm not exactly

sure where in the abdomen the liver resides, so I'm perhaps not the best person to ask!

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The Journal of Invasive Cardiology, Vol. 17, July 1, 2005,
entitled, "Tissue Engineering and Interventional Cardiology"
cited by the Examiner in the October 2, 2008 Office Action
issued in co-pending application Serial No. 09/836,750
(incomplete copy)

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Tissue Engineering and Interventional Cardiology

VOLUME: 17 PUBLICATION DATE: Jul 01 2005

Issue Number:
7

author:

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Murry et al. 1996 publication in <u>J. Clin. Invest.</u> entitled,
"Skeletal Myoblast Transplantation for Repair of Myocardial
Necrosis" cited by the Examiner
in the November 28, 2003 Office Action issued
in co-pending application Serial No. 09/836,750

Skeletal Myoblast Transplantation for Repair of Myocardial Necrosis

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Abstract

Myocardial infarcts heal by scarring because myocardium cannot regenerate. To determine if skeletal myoblasts could establish new contractile tissue, hearts of adult inbred rats were injured by freeze-thaw, and $3–4.5 \times 10^6$ neonatal skeletal muscle cells were transplanted immediately thereafter. At 1 d the graft cells were proliferating and did not express myosin heavy chain (MHC). By 3 d, multinucleated myotubes were present which expressed both embryonic and fast fiber MHCs. At 2 wk, electron microscopy demonstrated possible satellite stem cells. By 7 wk the grafts began expressing β-MHC, a hallmark of the slow fiber phenotype; coexpression of embryonic, fast, and β-MHC continued through 3 mo. Transplanting myoblasts 1 wk after injury yielded comparable results, except that grafts expressed β-MHC sooner (by 2 wk). Grafts never expressed cardiac-specific MHC-α. Wounds containing 2-wk-old myoblast grafts contracted when stimulated ex vivo, and high frequency stimulation induced tetanus. Furthermore, the grafts could perform a cardiac-like duty cycle, alternating tetanus and relaxation, for at least 6 min. Thus, skeletal myoblasts can establish new muscle tissue when grafted into injured hearts, and this muscle can contract when stimulated electrically. Because the grafts convert to fatigue-resistant, slow twitch fibers, this new muscle may be suited to a cardiac work load. (*J. Clin. Invest.* 1996. 98:2512–2523.) Key words: myocardial infarction • skeletal myoblast • myosin heavy chain • contractile function • cell transplantation

Introduction

Experimental and clinical therapies for myocardial infarction have focused traditionally on limiting infarct size. Unfortunately, the goal of limiting myocardial injury has been difficult to achieve clinically, because ischemic myocardium dies quite rapidly (1) and most patients wait more than 3 h after coronary occlusion before seeking medical attention. As an alternative approach, we are exploring strategies to induce the injured heart to heal with muscle replacement rather than forming scar tissue.

One strategy for muscle regeneration is to transplant either skeletal or cardiac myocytes into the injured heart. Studies from Field's group showed that cardiac myocytes can be transplanted into normal hearts, where they couple with host cardiocytes via intercalated discs (2, 3). However, a major drawback to using cardiocytes is their inability to proliferate in culture. At present it seems unlikely that enough primary cardiocytes could be obtained from the patient or histocompatible donor to repair a myocardial infarct in humans. On the other hand, skeletal muscle satellite cells (muscle stem cells) proliferate well in culture. Satellite cells could be obtained from muscles of infarct patients and rapidly expanded in culture, or stocks of potentially therapeutic myoblasts could be obtained from embryos and frozen for subsequent use (4, 5). Furthermore, physiological studies have shown that when properly conditioned, skeletal muscle can adapt to perform a cardiac-type work load (6). Recent studies have demonstrated the feasibility of grafting skeletal myoblast lines into normal hearts (7) and autologous satellite cells into injured hearts (8, 9). However, to generate significant amounts of functional new muscle the transplanted cells ideally should proliferate and then differentiate into mature myofibers capable of sustaining a cardiac work load. This study was performed to determine the proliferation and differentiation patterns of skeletal myoblasts after engraftment into injured rat hearts and to determine whether this new muscle could support contractile activity.

Methods

Skeletal myoblast isolation and culture. These studies were approved by the University of Washington Animal Care Committee and were conducted in accordance with federal guidelines. Skeletal myoblasts were obtained from the limbs of 1–3-d-old Fischer rats. This inbred strain was used to avoid immune barriers to transplantation. After time of killing, the carcasses were skinned and the limbs were placed into cold tissue culture media. Under a dissecting microscope, the muscles were stripped of surrounding adipose tissue and fascia and bluntly dissected from their tendons. The muscles were minced with iridectomy scissors until a fine slurry was formed. The slurry was then digested in 0.05% trypsin/EDTA (GIBCO-BRL, Gaithersburg, MD) in Ham's saline A at 37°C, with intermittent mechanical agitation to assist dispersal. After 30–45 min the cell suspension was filtered through sterile gauze to remove undispersed tissue fragments and rod shaped mature myofibers. Cells were plated at ~ 5×10^6 cells/dish in 100-mm gelatinized plates in 10 ml Ham's F10C media, containing 15% horse serum and 50 µg/ml gentamicin sulfate (Gemini Bioproducts, Inc., Calabasas, CA). Recombinant human basic fibroblast growth factor was added twice daily to a final concentration of 6 ng/ml, and the complete medium was replaced once per day. Approximately 10% of the cells attached and grew with a doubling time of ~ 18 h. The cultures contained a mix of small, oval myoblasts and elongated, spindle-shaped cells consistent with fibroblasts. Subconfluent cultures were passaged every 2–3 d (1:5 split) to minimize the occurrence of myogenic differentiation at higher density. On the day before transplantation, the cultures were tagged for subsequent identification in vivo. In some experiments cells were tagged with fluorescent micro-

spheres (1:500 dilution of stock 200 nm yellow-green fluorescent microspheres; Molecular Probes, Eugene, OR). The latex microspheres were endocytosed (typically > 20 spheres/cell) and served as cytoplasmic markers (10). In other experiments, cells were incubated overnight with [³H]thymidine (1 µCi/ml) to mark their nuclei after autoradiography. Cultures were trypsinized immediately before transplantation and suspended at a concentration of ~ 3 × 10⁷/ml. Small aliquots of the remaining cell suspension were replated at ~ 2 × 10⁴ cells/cm² into gelatinized, multichamber plastic slides, and fixed in methanol after various culture intervals for immunostaining.

Rat cardiac injury models. Inbred male Fischer rats (Simonsen Labs, Gilroy, CA) weighing 350–400 grams were anesthetized with intraperitoneal ketamine-xylazine (68 and 4.4 mg/kg, respectively), intubated, and mechanically ventilated with room air. The heart was exposed aseptically via a left thoracotomy, and a 1-cm-diameter aluminum rod, precooled with liquid nitrogen, was placed in direct contact with the anterior left ventricle for 15 s. Freeze-thaw reproducibly caused a disc-shaped region of coagulation necrosis, ~ 1 cm in diameter, extending ~ 2 mm into the myocardium. It should be noted that while infarcts typically have irregular borders with viable peninsulas of subepicardial myocardium along penetrating vessels, freeze-thaw lesions consist of confluent necrosis in the subepicardium with thaw lesions consist of confluent necrosis in the subepicardium with viable myocardium in the subendocardium. Because they are not transmural, freeze-thaw lesions do not cause cardiac aneurysm formation. Despite these differences, the cellular patterns of coagulation necrosis, inflammation and phagocytosis, granulation tissue formation, and scarring after freeze-thaw injury are indistinguishable from myocardial infarction (11–13), making it a suitable model to study myocardial repair.

In the initial studies, ~ 3 × 10⁶ myoblasts in 100 µl tissue culture media were injected superficially into the center of the injured region immediately after injury, using a 27-gauge needle. Then, the chest was closed and the rats were allowed to recover for timed intervals from 1 d to 3 mo (n = 4/time point). To mimic a clinical situation more closely, a second protocol was used in which the freeze-thaw lesion was allowed to heal for 1 wk before transplanting myoblasts. By 1 wk, most of the necrotic myocardium had been replaced by granulation tissue, but scar formation had not yet begun. The rats (n = 2/time point; no 3 d or 3 mo time points) were reanesthetized and a thoracotomy was repeated. The heart was exposed and a 100-µl suspension containing ~ 3 × 10⁶ myoblasts was injected into the wound as described above. The chest was closed and the animals were allowed to recover for intervals from 1 d to 7 wk.

To detect DNA synthesis in the grafts the rats were treated with the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU)[1] (Boehringer-Mannheim, Indianapolis, IN). 1 d before time of killing, the rats were lightly anesthetized, and a 50-mg tablet of BrdU was implanted subcutaneously for measurement of cell replication. Preliminary studies showed that a subcutaneous 50-mg BrdU tablet gave comparable replication rates to a 24-h continuous infusion with an osmotic mini-pump (not shown). For rats killed 1 d after transplantation, a single 10-mg pulse of BrdU was given intraperitoneally 1 h before time of killing. This avoided incorporation of BrdU into the cells which were cycling at the time of transplantation.

Rats were killed with a pentobarbital overdose and their hearts were excised. In the immediate transplantation groups, the aorta was cannulated and the hearts were perfusion fixed with methyl Carnoy's solution (60% methanol, 30% chloroform, 10% glacial acetic acid), transversely sectioned, and embedded in paraffin by routine methods. In groups transplanted 1 wk after injury, the hearts were transversely sectioned, embedded in OCT (Miles Inc., Kankakee, IL), and frozen in a dry ice-ethanol bath for frozen section analysis. In both protocols, sections of gut were obtained as controls for measurement of cell replication with BrdU.

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1. *Abbreviations used in this paper:* BrdU, 5-bromo-2'deoxyuridine; MHC, myosin heavy chain.

Measurement of contractile function in isolated wound strips. Rat hearts were given 4.5 × 10⁶ myoblasts (n = 8) in 100 µl or a sham injection of saline (n = 3) immediately after injury. 2 wk after engrafting, the hearts were excised and transversely sectioned. Under a dissecting microscope, most of the subendocardial myocardium was trimmed away from the injured region, and isolated wound strips (~ 1.5 × 1.5 × 8 mm) were prepared. One or two strips were studied from each myoblast-engrafted heart, and two or three strips were studied from each sham-injected heart. The strips were ligated at both ends with silk suture and then placed in a bath of physiological saline with the following composition (mmol/liter): 116 NaCl, 4.6 KCl, 1.2 MgSO₄, 2.5 CaCl₂, 26 Mops (pH 7.4), 11 glucose, and 10 mg/liter gentamicin. The buffer was equilibrated with 95% O₂/5% CO₂ and maintained at 20°C via a thermostatically controlled water jacket. Wound strips were mounted between an isometric force transducer (model 60-2995; Harvard Apparatus, Inc., South Natick, MA) and a fixed glass hook. Resting tension was set initially at 0.5 g. Strips were stimulated with 1-ms bipolar pulses delivered via platinum wire electrodes using a Grass model S48 stimulator (Astro-Med. Inc., West Warwick, RI). Voltage was increased in 10-V increments until contractile activity was observed. Force traces were displayed on a digital storage oscilloscope (model 3091; Nicolet Instrument Corp., Madison, WI) and recorded using a General Scanning model RS4-5P strip chart recorder. After determining the force–voltage relationship, the optimal length for force production was determined for each wound strip using test contractions at 2-min intervals, a time sufficient for metabolic recovery in mammalian fast twitch muscles (14). Force–frequency analysis was performed by increasing the stimulation frequency in 1-Hz increments; tetanus was defined as the point where the oscillations of contractile force at the plateau were < 3% of the net force generated (14). Finally, to test fatigability the grafts were subjected to a simulated cardiac-like duty cycle, consisting of 0.33 s of tetanus followed by 0.67 s of relaxation (1:2 cycle), continuing for 6 min. After completion of functional studies the strip's cross-sectional area was determined, and the tissue then was processed for histology or electron microscopy.

Immunocytochemistry. Antibodies used for immunostaining are given in Table I. 6-µm frozen sections were cut on a cryostat, briefly air dried, and stored at −70°C until use. 5-µm paraffin sections were deparaffinized in xylene and rehydrated in a graded alcohol series. Cultured cells were fixed and stored in cold PBS until use. For all samples, endogenous peroxidase activity was quenched by incubating with 0.3% H₂O₂ in methanol for 30 min. Immunostaining was carried out at room temperature. Sections were blocked with 1.5% normal out at room temperature. Sections were blocked with 1.5% normal horse serum in PBS for 1 h. The sections were then incubated with the primary antibody in 1.5% horse serum for 1 h, followed by incubation with the secondary antibody (rat adsorbed horse anti–mouse, 1:400 dilution; Vector Labs. Inc., Burlingame, CA) for 1 h. Antigens were localized with an avidin-biotin-peroxidase complex (ABC Elite kit; Vector Labs). For staining with a single antibody, diaminobenzidine (Sigma Immunochemicals, St. Louis, MO) was used as a chromadine (Sigma Immunochemicals, St. Louis, MO) was used as a chromagenic substrate. For double immunolabeling with antibodies to myosin and BrdU, sections were first exposed to 1.5 N HCl for 15 min at 37°C to denature the DNA, followed by a rinse in 0.1 mol/liter borax to stabilize the denatured strands. Sections were then stained routinely for myosin heavy chain (MHC) using diaminobenzidine. After a second quenching in 0.3% H₂O₂, sections were blocked with 1.5% normal horse serum, and then incubated with a mouse monoclonal antibody to BrdU for 1 h. After incubation with the secondary antibody (horse anti–mouse), BrdU was localized with an avidin-biotin-peroxidase complex, using True Blue (KPL, Gaithersburg, MD) as substrate. Cross-reactivity between the first primary antibody and the second secondary antibody did not occur, as long as the True Blue substrate was incubated for a short duration (< 1 min). Sections were counterstained either with methyl green, nuclear fast red, or hematoxylin.

Electron microscopy. After measurement of contractile function, one of the tissue strips was immersed in half strength Karnovsky's fix-

Antibody	Antigen recognized	Dilution	Source	Reference
MF-20	Sarcomeric MHCs	Hyb. Sup., 1:100	American Type Culture Collection, Rockville, MD	39
MY-32	Skeletal MHC-fast (types IIA and IIB)	Mouse ascites, 1:2000	Sigma Immunochemicals	40
BA-G5	Cardiac MHC-α	Hyb. Sup., 1:5	American Type Culture Collection	41
F1.652	Embryonic MHC	Hyb. Sup., 1:100	Developmental Studies Hybridoma Bank*	42
A4.951	β-MHC	Hyb. Sup., 1:50	American Type Culture Collection	43
Anti-BrdU	BrdU	IgG, 1:50000	Eurodiagnostics, Apeldoorn, The Netherlands	44

ative and dissected into small cubes < 1 mm in greatest dimension. The tissue was fixed overnight in half strength Karnovsky's fixative, postfixed for 1 h in 1% osmium tetroxide at room temperature, dehydrated through a graded alcohol series followed by propylene oxide, and embedded in Medcast resin (Ted Pella, Inc., Redding, CA). Semithin sections were stained with toluidine blue and examined by light microscopy. Thin sections were cut from selected blocks, stained with lead citrate and uranyl acetate, and examined in a Jeol JEM 1200EXII transmission electron microscope. Representative areas were photographed.

Results

Characteristics of myoblast cultures. The muscle cultures contained a mixed cell population. At least 22% of the cells were skeletal muscle, as indicated by their staining for sarcomeric myosin after switching to a differentiation medium containing 1.5% serum and no FGF for 3 d. This procedure underestimates the true percentage of skeletal muscle cells by severalfold, since the nonmyogenic cells continue to divide after the medium switch while the myoblasts complete their present cell cycle and then terminally differentiate. Approximately 1% of the cells stained with antibodies to smooth muscle α-actin, which can mark either smooth muscle cells or fibroblasts. Virtually none of the cells stained with an antibody for the endothelial marker von Willebrand factor. The remaining cells were presumably fibroblasts.

Histology and differentiation patterns of myoblast grafts. Cultured skeletal myoblasts were transplanted into cardiac freeze-thaw lesions either immediately after injury, or, to mimic a clinical situation more closely, cells were transplanted 1 wk after injury. The two protocols yielded similar results and will be described together; minor differences are noted below. On the first day after transplantation the myoblasts were mononuclear cells (Fig. 1 A). The grafted cells could be distinguished clearly from inflammatory cells within the necrotic tissue by their larger size and characteristic oval shape. (Fibroblast ingrowth from the surrounding tissue had not yet begun at this time.) The identity of the grafted cells was confirmed by their cytoplasmic fluorescent microspheres and radioactive nuclei (not shown). Mitotic figures were common. The grafted cells did not stain with antibodies to skeletal or cardiac MHCs (Fig. 1 B). Thus, muscle differentiation had not yet occurred.

By 3 d after transplantation, many of the grafted cells had fused to form multinucleated myotubes (Fig. 1 C). Myotubes were partially aligned along the short (transverse) axis of the

heart. The myotubes stained with antibodies to sarcomeric MHC, embryonic MHC (Fig. 1 D), and to MHC-fast (not shown). Occasional cross-striations were noted, but these were not frequent at this time (Fig. 1 D). The myotubes did not express cardiac MHC-α. By 1 wk the grafts were easily recognizable as skeletal myofibers and many cells contained cross-striations. As before, the new myofibers stained with antibodies to sarcomeric MHC, embryonic MHC, and MHC-fast, but did not express cardiac MHC-α (not shown). By 2 wk after transplantation the grafts had the appearance of maturing skeletal myofibers (Fig. 1 E). Sarcomeres were well formed, and many cells had peripheral nuclei. The myofibers stained intensely with antibodies to sarcomeric myosin, embryonic MHC (Fig. 1 F), and skeletal MHC-fast (Fig. 1 G). No staining with cardiac MHC-α antibodies was observed at 2 wk.

At 7 wk after transplantation the grafts were islands of mature skeletal muscle within young scar tissue (Fig. 1, H-J). There was a moderate increase in cell diameter compared with 2 wk. None of the muscle grafts were infiltrated or splayed apart by scar tissue, nor was there evidence of fiber atrophy. Vascular density appeared normal for muscle tissue (Fig. 1 J). All of the 7-wk grafts stained strongly with antibodies to sarcomeric myosin and embryonic MHC (Fig. 1 H). The grafts injected immediately after injury stained intensely with antiskeletal MHC-fast, comparable with Fig. 1 F. In contrast, the grafts injected 1 wk after injury stained poorly with antiskeletal MHC-fast (see below). No staining with antibodies to cardiac MHC-α was observed in the grafts, while the adjacent myocardium stained intensely (Fig. 1 I).

At 3 mo after transplantation the grafts again had the appearance of mature skeletal muscle (Fig. 1 K). Most myofibers had peripheral nuclei, and vascular density appeared normal. Fiber diameter was generally larger than in the 7-wk group, indicating that the cells had hypertrophied between 7 wk and 3 mo (compare Fig. 1, J and K). In one heart, however, part of the graft was infiltrated by scar tissue which encircled individual myofibers and was associated with fiber atrophy (Fig. 1 L). The grafts continued to express embryonic MHC and MHC-fast (not shown). Once again, no staining with antibodies to cardiac MHC-α was observed (comparable with Fig. 1 I). At all time points the myofibers were predominantly aligned parallel with the short (transverse) axis of the heart and therefore appeared in longitudinal section. However, some fascicles of muscle appeared obliquely or cross-sectioned in this plane.

In summary, the grafts began to differentiate into myo-

Figure 1. Morphology and MHC expression in skeletal myoblast grafts. Rat hearts were injured by freeze-thaw and syngeneic skeletal muscle cells were grafted into the lesions. All panels in this figure are from hearts which were grafted immediately after injury. (*A*) 1-d graft. The grafted cells are identifiable as relatively large, oval shaped cells (*arrows*) within the necrotic myocardium. One graft cell is in mitosis (*arrowhead*). Numerous smaller inflammatory cells are present within the lesion. Fibroblast ingrowth from surrounding viable tissue had not yet begun at this

tubes between 1 and 3 d and acquired the appearance of maturing myofibers with well formed sarcomeres by 2 wk. The grafts expressed both embryonic MHC and MHC-fast at all times between 3 d and 3 mo. There was no expression of cardiac MHC-α at any time.

Electron microscopy. Electron microscopy was performed on one heart, 2 wk after myoblast grafting. Most of the grafted cells had well formed, slightly contracted sarcomeres which were aligned in registry (Fig. 2 *A*). Mitochondria were abundant. Multinucleation was evident in many cells, as were well formed T-tubules. However, in other cells a spectrum of morphological stages was present, consistent with developing skeletal muscle (Fig. 2 *B*). Cells at the earliest stage were small, had scanty myofibril content, and contained focal aggregations of electron-dense material suggestive of developing Z-discs. In these cells there were abundant ribosomes and glycogen, a prominent Golgi apparatus, and dilated segments of sarcoplasmic reticulum. Intermediate cells were larger and had increasing amounts of myofibrils with a corresponding decrease in ribosomes and glycogen. Some cells had well formed sarcomeres, but these were out of registry compared with the most mature cells. No intercalated discs were identified between cells in the graft region. Adjacent myofibers often had intimately apposed, interdigitating cell membranes. Occasional cells were identified with electron-dense membrane structures suggestive of intermediate adherens junctions and gap junctions (Fig. 2, *C* and *D*). Some mature myofibers were closely associated with mesenchymal cells, located within the basal lamina compartment of the myofiber. Their location within the basal lamina of the myofiber suggests that they might be new satellite stem cells (Fig. 2, *E* and *F*). Some of these mesenchymal cells had abundant rough endoplasmic reticulum, similar to fibroblasts. Cells with this morphology have also been described in regenerating skeletal muscle by Trupin et al. (15). Their location within the basal lamina of the myofiber and the

absence of collagen in this space make it unlikely that these cells are actually fibroblasts.

Myoblast grafts convert from fast to slow twitch fibers. The poor staining for MHC-fast in the 7-wk group with delayed transplantation seemed at variance with the morphology of the grafts, which showed relatively hypertrophic cells with well formed sarcomeres. We hypothesized that the grafts had undergone fiber type conversion to slow twitch muscles, which no longer expressed high levels of MHC-fast. Slow twitch fibers have physiological similarities to cardiac muscle, including a high capacity for oxidative phosphorylation and fatigue resistance. Furthermore, slow fibers use β-MHC as a major contractile protein, which is also the predominant myosin in developing rat hearts. In contrast, fast twitch fibers use glycolysis for ATP production, have a low aerobic capacity and fatigue rapidly, and do not express β-MHC (16). Therefore, we compared β-MHC expression with skeletal MHC-fast, to determine fiber types in the maturing grafts.

At 1 wk the grafts stained intensely for MHC-fast (Fig. 3 *A*) but did not stain with an antibody to β-MHC (Fig. 3 *B*). At 2 wk the grafts continued to express MHC-fast. In the group transplanted immediately after injury no expression of β-MHC was noted at 2 wk, yet in grafts transplanted 1 wk after injury some cells expressed β-MHC (not shown). At 7 wk after transplantation the two groups differed in expression of MHC-fast, with strong staining in the immediate transplant group (see Fig. 1 *G*) and weak staining in the group where transplantation was delayed for 1 wk after injury (Fig. 3 *C*). However, both the immediate and delayed transplantation groups exhibited extensive staining for β-MHC at 7 wk after transplantation (Fig. 3 *D*). At 3 mo there was continued expression of β-MHC and MHC-fast in the immediate transplantation group; we did not study the delayed transplantation protocol at 3 mo. Thus, myoblast grafts appeared to be undergoing conversion from fast twitch to slow twitch fibers. Conversion appeared to take place

Figure 2. Transmission electron micrographs of 2-wk-old myoblast graft. The graft was placed immediately after cardiac freeze-thaw injury. (*A*) Low magnification overview showing well differentiated, striated skeletal myofibers (*MF*) within a collagen-rich matrix. A small venule (*V*) is shown at the left aspect. *Nu*, nucleus. Bar, 5 μm. (*B*) Moderately differentiated skeletal myofiber containing two nuclei (*Nu*). a modest complement of myofibrils (*mf*), and abundant ribosomes and sarcoplasmic reticulum between the nuclei. The sarcolemma is delimited by arrowheads. Bar, 1 μm. (*C*) Intercellular junction formation between adjacent myofibers. The two cells have closely apposed and interdigitated membranes. Two electron-dense plaques between the cells are present within the boxed region, suggestive of an adherens type intermediate junction and a gap junction, shown at higher magnification in *D*. Bar, 1 μm. (*D*) Higher magnification of the junctional region boxed in *C*, showing putative intermediate junction between adjacent myofibers (*solid arrow*) and gap junction (*open arrow*). Bar, 0.5 μm. (*E*) Skeletal myofiber (*MF*) with closely apposed mesenchymal cell atop it, suggestive of a satellite cell (*SC*). The boxed region is shown at higher magnification in *F*. Bar, 2 μm. (*F*) Higher magnification of region boxed in *E*. The putative satellite cell (*SC*) and the myofiber (*MF*) are contained within the same basal lamina compartment (*BL*, outlined by *arrowheads*). Although the cell has abundant rough endoplasmic reticulum, its location within the basal lamina of the myofiber and the absence of fibrillar collagen from this space make it unlikely that this is a fibroblast. Bar, 1 μm.

more rapidly when cells were transplanted into an injury with more advanced healing.

Proliferation of myoblast grafts. To identify cells undergoing DNA synthesis. the thymidine analogue BrdU was admin-

istered for 24 h before time of killing in most groups; animals in the day 1 group received a single pulse of BrdU 1 h before time of killing. Double immunostaining was performed with antibodies to the fast isoform of MHC and to BrdU, to detect

Figure 3. Fiber type conversion and proliferation of engrafted skeletal myoblasts. For the fiber typing experiments, rat hearts were injured by freeze-thaw and the lesions were allowed to heal for 1 wk. Syngeneic skeletal myoblasts were engrafted into the 1-wk-old wounds. For studies of cell proliferation, myoblasts were engrafted immediately after cardiac injury. Rats were killed at the indicated times after transplantation. Antibodies specific to fast twitch (MHC-fast) and slow twitch (β-MHC) fibers were used to define fiber types. Processing for frozen sections in *A–D* resulted in formation of contraction bands, artifactual clumping of the sarcomeres due to hypercontracture. BrdU was administered 24 h before time of killing to detect DNA synthesis. Double immunostaining for BrdU and MHC was then performed on paraffin sections. Appearance of a BrdU-positive nucleus within a myosin-positive cell indicated the myoblast had replicated and fused into the myotube within the last 24 h. (*A*) 1-wk graft stained for fast fiber isoforms of MHC. There is intense staining of the engrafted cells, indicating a fast fiber phenotype. Hematoxylin counterstain. ×960. (*B*) 1-wk graft stained with an antibody to the slow fiber-specific β-MHC. None of the grafted cells express β-MHC at this time, indicating that the cells show no characteristics of slow fibers. Methyl green counterstain. ×960. (*C*) 7-wk graft stained with an antibody to MHC-fast. There is weak staining compared with the 1-wk graft (*A*). Methyl green counterstain. ×960. (*D*) 7-wk graft stained with an antibody to β-MHC. The grafted cells now express β-MHC, indicating that they are acquiring a slow fiber phenotype (compare with *B*). Methyl green counterstain. ×960. (*E*) 3-d graft doubly stained for BrdU (purple) and MHC-fast (brown). One nucleus within the myotube stains purple (*arrow*), indicating it has undergone DNA replication before fusion into the myotube. The remaining nuclei in the myotube do not contain BrdU and pick up the red counterstain. Numerous myosin-negative cells in the surrounding wound tissue also stain positively for BrdU. Nuclear fast red counterstain. ×2,400.

A

Twitch   4.0 Hz   8.0 Hz   Control

(y-axis: grams/mm², values 0.0, 0.4, 0.8)

Time (in sec)

(x-axes: 0.0 4.0 8.0; 1.0 for Twitch)

B

(y-axis: % Maximal Force, 0 to 100; x-axis: Voltage, 10 to 120)

C

(y-axis: % Initial Force, 0 to 100; x-axis: Time (min), 0 to 6)

Figure 4. Contractile function of myoblast grafts ex vivo. Wound strips from injured hearts receiving either skeletal myoblasts or a sham saline injection were excised at 2 wk. Wounds were mounted on an isometric tension myograph in oxygenated buffer and electrically stimulated. The carats indicate the onset of electrical stimulation. Force has been normalized to cross-sectional area. (A) The first panel shows individual muscle twitch in a myoblast-injected wound. Note the rapid contraction and relaxation rates. The second panel shows that individual twitches began to superimpose with a stimulation frequency of 4 Hz, with a resulting potentiation in developed tension. The third panel shows that tetanus was induced with stimuli ≥ 7 Hz. Note the further increase in tension compared with the 4-Hz stimulation. Time to peak force in this preparation was ~ 1 s, faster than was typically observed for the overall group. The fourth panel shows that no tension was developed at any voltage in a sham-injected wound. This tracing is representative of six wound strips from three sham-injected hearts. (B) Force–voltage relationship. Developed tension for individual twitches increased as stimuli increased from 30 to 100 V, indicating recruitment of additional myofibers. Data have been normalized to maximal developed tension and are presented as mean±SEM of eight wound strips from six hearts. (C) Fatigue test. Wounds containing myoblast grafts were subjected to a cardiac-like duty cycle, consisting of repeated episodes of 0.33 s of tetanus/0.67 s of rest, to mimic a heart rate of 60 beats/min. There was a 53% decrease in developed tension at the end of the 6-min test. Note that most of the diminution in force occurred during the first 3 min. Data represent mean±SEM of seven wound strips from five hearts.

myoblasts which had proliferated and subsequently differentiated. In the day 1 grafts, proliferating cells were present within the necrotic lesion, which could have represented either graft cells or macrophages. As mentioned above, none of the cells expressed MHC at this time, so it was not possible to determine which among these were myoblasts (versus transplanted fibroblasts or host macrophages). In the day 3 grafts, occasional BrdU-positive nuclei were identified within myosin-pos-

itive cells (Fig. 3 *E*). We observed a total of 12 such nuclei in three hearts. No attempt was made to quantify this low rate, but it was certainly < 1% of total nuclei in myosin-positive cells. Virtually no BrdU-positive nuclei were seen in myosin-positive cells at 1, 2, or 7 wk after transplantation (not shown). We conclude that myoblast proliferation occurs for at least 3 d after grafting, but by 1 wk virtually all cells have ceased replicating.

Contractile function of myoblast grafts. The contractile properties of 2-wk-old myoblast grafts were determined by attaching isolated wound strips to a tension myograph ex vivo. Virtually no spontaneous mechanical activity was detected, consistent with the paucity of cardiomyocytes histologically. Electrical stimulation caused muscle twitches in six of eight myoblast-engrafted hearts (Fig. 4 *A, first panel*); strips from the remaining two hearts may have been damaged during sample preparation, since skeletal muscle was present histologically. The grafts showed a stepwise increase in tension development as voltage was increased from 30 to ~ 100 V with a plateau thereafter (Fig. 4 *B*). This indicates that increasing voltage recruited additional myofibers to contract, implying that the graft myofibers are electrically insulated from one another. It should be noted that cardiac muscle does not increase contractile force with increasing voltage, since cardiocytes are coupled electrically via gap junctions.

Next, force–frequency relationships were determined. Using 120% of the voltage required for maximal tension development, the frequency of stimulation was increased incrementally from 0.5 to 10 Hz. Twitches began to superimpose at frequencies of 3–4 Hz, with a resulting increase in total developed tension (Fig. 4 *A, second panel*). Fully fused tetani were produced with 6–7 Hz stimulation (Fig. 4 *A, third panel*). Peak force during tetanus was 1.98±0.45 grams (mean SEM); after normalization to cross-sectional area the peak force was 0.72±0.14 grams/mm². The time to peak tetanic force averaged 2.3±0.3 s, although 90% of peak force was typically generated within 1.5 s. The time to half-relaxation after tetanus was 240±17 ms. It should be stressed that tetanus cannot be induced in cardiac muscle, due to the long refractory period of cardiocytes.

Finally, a fatigue test was performed to test the response of this muscle to a cardiac-like work load. The grafts were subjected to a duty cycle consisting of repeated 0.33 s of tetanic stimuli followed by 0.67 s of rest, mimicking a heart rate of 60 beats/min. The grafts showed a 32% decline in developed tension by 2 min and a 53% decline by the end of the 6-min test period (Fig. 4 *C*). No contractile activity could be elicited from six of seven wound strips from three injured hearts which received a sham injection of saline instead of myoblasts (Fig. 4 *A, fourth panel*). In one sham heart an adhesion had developed between the heart and chest wall, resulting in a small amount of intercostal muscle adhering to one of the two wound strips. In this preparation we detected a peak force of 0.04 grams/mm², < 2% of what was present in the myoblast-engrafted hearts.

Thus, the skeletal muscle grafts could be stimulated to contract ex vivo and could sustain a cardiac-like duty cycle over a 6-min test period. Furthermore, the grafts showed two physiological properties unique to skeletal muscle: recruitment of fibers with increasing voltage and the ability to sustain tetanic contraction. We do not know yet whether the grafts contract in vivo.

Discussion

The principal findings of this study are: (*a*) neonatal skeletal myoblasts can be grafted into an injured heart; (*b*) the engrafted myoblasts initially proliferate and then begin to form multinucleated myotubes by day 3; (*c*) the myotubes differentiate into mature myofibers, which initially have a phenotype similar to fast twitch muscle; (*d*) the myofibers develop characteristics of slow twitch muscle as the wound heals; (*e*) the new muscle may form satellite stem cells; and (*f*) the new muscle can be stimulated to contract ex vivo.

Strategies for muscle regeneration after myocardial injury. In principal, there are at least three strategies to induce muscle regeneration after myocardial infarction. First, the surrounding cardiac myocytes could be stimulated to migrate into the wound and proliferate to repair the defect. There is evidence that a limited amount of cell replication by adult cardiocytes occurs naturally after myocardial infarction in humans (17) and in rats (18, 19), but the response is clearly not adequate to repair the defect. The factors responsible for cell cycle arrest in cardiocytes are not well enough defined at present to begin exploring this as a therapy. (The interested reader is referred to references 20–23 for further information on this topic.)

A second strategy is to induce the cells of cardiac granulation tissue (the fibroblast-rich tissue of wound repair) to differentiate into muscle rather than forming a scar. There is not enough known about cardiac differentiation at present to attempt formation of new myocardium. However, much more is known about skeletal muscle determination. The discovery of myogenic determination genes (24, 25) has made it possible to induce a wide range of cultured cell types to differentiate into skeletal muscle. Recent studies from our group (26) and others (27) have shown that cells in cardiac granulation tissue can be induced to differentiate into skeletal muscle by transfection with the prototype myogenic determination gene, MyoD. In these early experiments, however, the frequency of muscle differentiation has been low after MyoD gene transfer. Until the frequency of myogenic conversion can be increased, it will be difficult to investigate the functional properties of the MyoD-induced skeletal muscle.

The third strategy for muscular repair of infarcts is to transplant either skeletal or cardiac myoblasts into the injured region. Studies by Koh et al. (3) and Soonpaa et al. (2) have demonstrated that fetal cardiocytes will form intercalated discs with host cardiocytes, including gap junctions and adherens junctions, when transplanted into normal hearts. No proliferation was detected in the grafted cardiocytes. Less information is available on grafting of cardiocytes into injured hearts. Our group (28) and others (29, 30) have preliminary data showing that neonatal rat or fetal human cardiocytes can be transplanted successfully into injured rat hearts. To our knowledge there is no information regarding proliferation of these grafts, nor are any functional data available. As discussed above, the principal limitation to this approach is the inability to induce cardiocytes to proliferate in culture. Until cardiocytes can replicate in vitro, or proliferation-competent cells can be induced reliably to differentiate into cardiocytes, cardiocyte grafting will not be feasible in humans.

In contrast to cardiocytes, proliferating skeletal muscle precursors are readily available, either as primary myoblasts in developing muscle or as satellite cells from quiescent muscle. In this study six rat pups yielded the myoblasts implanted into

27 injured hearts. In addition to their growth in culture, the myoblasts proliferated in vivo for several days after transplantation (Fig. 3 E). These properties have led us and several other groups to explore skeletal muscle grafting for cardiac repair. Koh et al. (7) demonstrated that the myogenic cell line C2C12 could be transplanted into the hearts of normal syngeneic mice, where the cells fused to form multinucleated myofibers. The same group also demonstrated that C2C12 cells stably transfected with a plasmid encoding active TGF-β could induce angiogenesis around the graft site (31). No coupling between the host cardiocytes and the grafted skeletal muscle was observed in either experiment.

Chiu et al. (8, 9) transplanted autologous satellite cells into cardiac freeze-thaw lesions in dogs. Comparable with our study, they also found that the grafts formed muscle cells within the healing lesion. In distinction to the current study, however, they hypothesized that their grafted skeletal muscle cells differentiated into cardiac muscle, via "milieu-dependent effects." The evidence for a cardiac phenotype was that some cells within the grafts had central rather than peripheral nuclei, and some cells contained refractile transverse structures under light microscopy interpreted to be intercalated discs. Although we observed some myofibers with persistent central nuclei in this study, as well as rare cells showing intermediate and gap junctions (Fig. 2, C and D), no intercalated discs were present by electron microscopy. More importantly, the grafted cells expressed skeletal muscle–specific proteins and failed to express the cardiac-specific isoform MHC-α up to 3 mo after transplantation. Thus, there clearly was no cardiac differentiation in this study.

Conversion of grafts from fast to slow twitch muscle. Although the skeletal muscle grafts expressed the fast fiber isoform of MHC at 1 and 2 wk, they expressed β-MHC, a marker for slow twitch fibers, at 7 wk and 3 mo. This indicates that the grafts were converting to slow twitch fibers. Conversion was apparently more rapid when the myoblasts were injected into wounds where healing had been allowed to progress for 1 wk, as opposed to immediately after injury. In the delayed transplantation model the grafts expressed β-MHC at 2 wk, while in the immediate transplantation model this protein was not detected until 7 wk. It is possible that the growth factors and cytokines present in the early wound delay myoblast differentiation and subsequent fiber type conversion.

Slow fibers exhibit several important differences from fast fibers, including a slower shortening velocity, use of oxidative phosphorylation for ATP production, a higher mitochondrial content, a higher myoglobin content, and a much greater resistance to fatigue (16, 32). An interesting parallel is that the latissimus dorsi muscle also undergoes fiber type switching when it is conditioned for dynamic cardiomyoplasty. Cardiomyoplasty is an experimental therapy for heart failure, where skeletal muscle is wrapped around the heart to serve as a ventricular assist device (33). Untrained latissimus dorsi is a mixed fiber type muscle which fatigues rapidly with repeated stimulation. When conditioned by repeated electrical stimulation for 6 wk before surgery, however, the latissimus dorsi converts entirely to slow twitch fibers and becomes fatigue resistant (6). Only the conditioned, slow twitch muscle is able to assist cardiac function. This parallel suggests the intriguing possibility that repeated electromechanical stimulation leads to activation of the slow fiber phenotype. Since we did not test whether the environment of the heart contributed to fiber type conversion,

additional experiments will be required to determine the mechanism. The fact that the grafts differentiated into slow twitch fibers suggests that they may be suited to perform a cardiac type work load.

Will skeletal muscle transplantation augment cardiac function? This study definitively showed that myoblast grafting can generate new contractile tissue. The skeletal muscle grafts exhibited characteristic twitches when stimulated ex vivo (Fig. 4 A) and showed recruitment of contractile units with increasing voltage (Fig. 4 B). Furthermore, tetanus could be induced with rapid stimulation (Fig. 4 A, second and third panels), and the grafts could perform a cardiac-like duty cycle for 6 min (Fig. 4 C). Peak force during tetanus averaged 0.7±0.1 grams/mm². Since the wound strips contained < 50% of the myofiber content of normal muscle, due to inclusion of scar tissue, the force can be normalized to at least 1.4 grams/mm² muscle. Adult mammalian muscle can generate 15–35 grams/mm² force at tetanus, depending on fiber type (14, 34). Thus, the 2-wk grafts generated ∼ 4–10% of the predicted force for mature skeletal muscle. Several factors may cause a lower than predicted force, including the relative immaturity of the 2-wk myofibers, stretching of the immature extracellular matrix, poor cell matrix attachments, or misalignment of some fibers relative to the axis of the wound strip.

Although preliminary, these results are encouraging and suggest that more detailed studies of contractile function are warranted in skeletal myoblast-engrafted hearts. A critical question is whether the skeletal muscle grafts contract in vivo. To provide coordinated mechanical assistance, the grafted cells ideally should form electrical and mechanical junctions with the host myocardium. In our grafts the skeletal muscle cells were insulated from the remaining myocardium by scar tissue, so there was no opportunity for myofiber–cardiocyte coupling to occur. Koh et al. (7) transplanted C2C12 myoblasts into normal mouse hearts and observed no cell junctions between grafted myofibers and host cardiocytes by electron microscopy. Although proliferating myoblasts have been reported to synthesize both gap junction proteins (35) and N-cadherin (36, 37), these proteins are typically absent from adult skeletal myofibers. By electron microscopy we observed evidence both for intermediate and gap junction formation between skeletal myofibers 2 wk after grafting (Fig. 2, C and D). This finding was infrequent, however, and it is unknown whether such junctions would persist in longer term grafts. If skeletal muscle will not couple spontaneously with cardiac muscle, it is possible that such junctions could be induced by stably transfecting skeletal muscle cells with genes for cardiac junctional proteins. Another possibility is that skeletal muscle grafts could be electrically paced in synchrony with the cardiac cycle. Pacing would require sufficient voltage to activate all of the fibers, and currently it is unknown whether this would have a deleterious effect on the surrounding myocardium.

In the uninjured heart there is a complex fiber geometry, where the outer fibers run in the long axis, the midwall fibers run in the short axis, and the inner fibers again run in the long axis. This geometry is established during embryogenesis and is thought to be important for mechanical efficiency. In this study, the grafted myofibers were predominantly aligned with the short (transverse) axis of the heart. Alignment was noted as early as day 3, when myotube formation was prominent (Fig. 1 C). This is the same orientation that wound fibroblasts and collagen fibers acquire during wound healing, and it seems

likely that all are aligned by local mechanical forces. It is not known whether alignment with the heart's short axis will influence the ability of these myofibers to restore mechanical function after injury.

There are two aspects of skeletal muscle which theoretically could make it superior to cardiac muscle for infarct repair. First, skeletal muscle is much more resistant to ischemia than cardiac muscle. Skeletal muscle can withstand many hours of severe ischemia without becoming irreversibly injured, whereas in myocardium irreversible injury begins within 20 min (38). A second difference is that skeletal myoblast grafts might establish satellite cells. Satellite cells are the resident stem cells in skeletal muscle and proliferate in response to injury. Once activated, satellite cells can fuse with damaged myofibers or establish new myofibers to replace those lost to necrosis. We observed cells within 2-wk grafts which were morphologically consistent with satellite cells by electron microscopy (Fig. 3, E and F). Thus, it is possible that infarcts repaired with skeletal myoblasts might become more resistant to a subsequent episode of ischemia or might be able to replace myofibers damaged by ischemia.

Acknowledgments

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EXHIBIT F

Dohmann, et al., 2005 publication in <u>Circulation</u>, entitled, "Transendocardial, Autologous Bone Marrow Mononuclear Cell Injection in Ischemic Heart Failure" cited in Examiner's Answer, dated November 28, 2007, in co-pending application Serial No. 09/794,456

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Heart Failure

Transendocardial Autologous Bone Marrow Mononuclear Cell Injection in Ischemic Heart Failure

Postmortem Anatomicopathologic and Immunohistochemical Findings

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▶ Abstract

Background— Cell-based therapies for treatment of ischemic heart disease are
currently under investigation. We previously reported the results of a phase I trial
of transendocardial injection of autologous bone marrow mononuclear (ABMM)
cells in patients with end-stage ischemic heart disease. The current report focuses
on postmortem cardiac findings from one of the treated patients, who died 11
months after cell therapy.

Methods and Results— Anatomicopathologic, morphometric, and immunocytochemical findings
from the anterolateral ventricular wall (with cell therapy) were compared with findings from the
interventricular septum (normal perfusion and no cell therapy) and from the inferoposterior
ventricular wall (extensive scar tissue and no cell therapy). No signs of adverse events were found in
the cell-injected areas. Capillary density was significantly higher ($P<0.001$) in the anterolateral wall
than in the previously infarcted tissue in the posterior wall. The prominent vasculature of the
anterolateral wall was associated with hyperplasia of pericytes, mural cells, and adventitia. Some of
these cells had acquired cytoskeletal elements and contractile proteins (troponin, sarcomeric α-actinin,
actinin), as well as the morphology of cardiomyocytes, and appeared to have migrated toward
adjacent bundles of cardiomyocytes.

Conclusions— Eleven months after treatment, morphological and immunocytochemical analysis of the sites of ABMM cell injection showed no abnormal cell growth or tissue lesions and suggested that an active process of angiogenesis was present in both the fibrotic cicatricial tissue and the adjacent cardiac muscle. Some of the pericytes had acquired the morphology of cardiomyocytes, suggesting long-term sequential regeneration of the cardiac vascular tree and muscle.

Key Words: angiogenesis • stem cells • heart failure • revascularization • ischemia

▶ Introduction

The role of cell-based therapy for the treatment of ischemic heart disease is currently under investigation. In view of the myocardium's limited capacity to regenerate spontaneously after an ischemic injury, the therapeutic use of exogenous progenitor cells has recently gained increasing interest. In vitro demonstration of functional cardiomyocyte differentiation from bone marrow–derived progenitor cells[1,2] has prompted in vivo studies in animal models, and promising results have been obtained in the repair and regeneration of acute and chronic cardiac muscle lesions. Several types of progenitor cells have been used in experimental models, including bone marrow–derived endothelial and blood cell progenitors, as well as bone marrow mesenchymal progenitors.[3–6]

In humans, similar attempts have been made with surgical, intracoronary, or transendocardial introduction of bone marrow–derived cells to improve cardiac lesions.[7,8] Our group recently reported the results of the first phase I human trial of transendocardial injection of autologous bone marrow mononuclear (ABMM) cells in patients with end-stage ischemic heart disease.[9] We observed a significant increase in perfusion, contractility of ischemic myocardial segments, and functional capacity of the cell-injection recipients. This report presents postmortem cardiac findings from one of these patients.

▶ Case Report

The patient was a 55-year-old man with ischemic cardiomyopathy and 2 previous myocardial infarctions (in 1985 and 2000). He began to have symptoms of congestive heart failure 2 years before study enrollment. One year before enrollment, the patient had an ischemic stroke with mild residual right hemiparesis

and resultant episodes of chi~.iic tonic-clonic seizures. His risk factors for coronary artery disease included diabetes mellitus type II, hypertension, and hypercholesterolemia.

The patient's functional capacity was evaluated at baseline by means of a ramp treadmill protocol[10] with a peak maximal oxygen consumption ($\dot{V}O_2max$) of 15.9 mL · kg^{-1} · min^{-1} and a workload of 4.51 metabolic equivalents (METs). A baseline single-photon-emission computed tomography (SPECT) perfusion study showed a partially reversible perfusion defect in the anterolateral wall, a fixed perfusion defect (scar) in the inferior and posterior walls, and normal perfusion in the septal wall.

Cardiac catheterization revealed a left ventricular ejection fraction of 11%, a 70% ostial and an 85% middle stenosis of the left anterior descending (LAD) coronary artery, an 80% proximal lesion of the left circumflex (LCx) coronary artery, and total occlusion of the first obtuse marginal artery and right coronary artery. The distal segments of the LAD and LCx were diffusely diseased. Owing to the severity and extent of the patient's coronary disease, he was not considered a candidate for surgical or interventional procedures. At enrollment in our study, he was in New York Heart Association (NYHA) functional class III and Canadian Cardiovascular Society (CCS) angina class III. His serum C-reactive protein level, complete blood count, creatine kinase level, and troponin level were normal at baseline.

The patient received a total of 3x10^7 ABMM cells (the Table) that had been harvested 2 hours before the procedure. With the guidance of electromechanical mapping,[11,12] the cells were injected transendocardially into the anterolateral wall of the left ventricle. No periprocedural complications were observed.

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Phenotype and Functional Characterization of 3x10^7 Cells Injected via a Transendocardial Route*

Noninvasive follow-up evaluation was performed 2 and 6 months after cell therapy. Invasive follow-up evaluation, with cardiac catheterization, was performed at 4 months and revealed no change in coronary anatomy. Symptomatic and functional improvements were noted because the patient returned to NYHA and CCS class I. Holter monitoring showed no malignant ventricular arrhythmias, and signal-averaged ECG parameters remained stable. There was no change in the patient's medications after cell therapy. There was no change in the global ejection fraction or left

ventricular volume on echocardiography. The wall-motion index score (on 2-dimensional echocardiography) improved from 1.94 to 1.65 as contractility increased in 5 segments adjacent to the injected area. Myocardial perfusion, as assessed by SPECT, improved in the anterolateral wall. Mechanical data derived from SPECT showed improvements in regional ejection fraction, wall motion, and thickening. In addition, during ramp treadmill testing, the $\dot{V}O_2$max increased from 15.8 to 25.2 mL · kg^{-1} · min^{-1}, and the METs increased from 4.51 to 7.21 at 2 months. At 6-month follow-up testing, the $\dot{V}O_2$max reached 31.6 mL · kg^{-1} · min^{-1}, and the METs was 9.03.

From 6 to 11 months after the cell injection procedure, the patient's cardiovascular condition remained stable. At 11 months, however, he had a tonic-clonic seizure at home and was found in cardiopulmonary arrest by family members.

▶ Methods

After signed, informed consent was given by the family, an autopsy was performed, including morphological and immunocytochemical analysis of the heart. This report presents the anatomicopathologic findings about the infarcted areas of the anterolateral ventricular wall, which were the areas that had received bone marrow cell injections. The histological findings from this region were compared with findings from within the interventricular septum (which had normal perfusion in the central region and no cell therapy) and findings from the previously infarcted inferoposterior ventricular wall (which had extensive scarring and no cell therapy).

Immunocytochemical analysis of paraffin sections was performed with antibodies against factor VIII–related antigen (A0082, Dako), vimentin (M0725, Dako), smooth muscle α-actin (M0851, Dako), and CD34 and Ki-67 (NCL-L-End and NCL-Ki67MM1 respectively, Novocastra). Antibodies were reacted with Dako's EnVision+ System/HRP, with diaminobenzidine as a chromogen. Frozen sections were fixed, permeated with acetone, and incubated with antibodies for troponin T (T6277, Sigma), smooth muscle α-actin, sarcomeric actinin (A7811, Sigma), and desmin (D1033, Sigma). Antibodies were revealed with anti-mouse or anti-rabbit IgG, F(ab)2 fragment, conjugated to fluorescein isothiocyanate (1814192 and 1238833, respectively, Boehringer-Mannheim), and counterstained with a 0.1‰ solution of Evans blue dye (Merck).

Capillary density was monitored by using computerized image analysis (Image-Pro Plus, MediaCybernetics) of randomly selected fields in sections stained with hematoxylin and reacted with antibody for factor VIII–related antigen (n=108) and randomly selected fields in sections reacted with antibodies for smooth muscle α-actin (n=96). Transverse sections of capillaries identified by staining

for factor VIII and pericyte-containing capillaries identified by staining for smooth muscle α-actin were quantified separately. Results were expressed as the mean number of capillaries per square millimeter in the case of factor VIII–stained slides or the number of capillaries containing pericytes in α-smooth-muscle-actin–stained slides. Larger vessels identified by a continuous wall of smooth muscle actin–positive mural cells were excluded. Differences between the anterolateral, septal, and posterior walls were assessed with Kruskal-Wallis ANOVA and the Student-Newman-Keuls method for pairwise multiple comparison. Results were considered significant if P was <0.05.

Evaluation of the capillary density inside the fibrotic areas within the cell-treated anterolateral wall versus the nontreated posterior wall was performed in 40 selected fields inside the fibrotic scars, excluding the regions containing cardiomyocytes. Microscope fields (at ×100) of factor VIII–stained slides were digitized, and the number of transverse sections of capillaries per square millimeter of fibrotic zones was assessed. Differences between the treated infarcted zones and the nontreated fibrotic wall were assessed by the Mann-Whitney rank-sum test. Results were considered significant if P was <0.05.

▶ Results

Anatomopathologic Findings

The heart weighed 765 g. There was severe arteriosclerosis with subocclusive calcified atheromata in all coronary arteries, calcification of the pulmonary artery, and moderate atheromatosis of the aorta. The heart cavities were dilated, with hypertrophic walls. There was no evidence of any acute injury or of lesions that could be related to cell injections. A generalized, homogeneous endocardial opacification, affecting all the cardiac internal surfaces, was identified on histological examination as diffuse fibroelastic hyperplasia of the endocardium. Minute focal and punctate scars were observed, mainly in the posterior and anterolateral walls.

The apical zone was thinned and fibrotic. The posterior and apical regions had dense, fibrotic, well-circumscribed scars that separated cardiomyocyte bundles. The septal wall exhibited focal scars interspersed with cardiac fibers in the regions adjacent to the anterior and posterior ventricular walls, but it was devoid of fibrosis in the central region.

The anterolateral ventricular wall that received cell injections had elongated, irregular, and parallel reddish areas throughout. In the same wall, in adjacent regions that did not receive injections, the density and morphology of the fibrotic scars were similar to those of the posterior wall, suggesting that no overt differences were present among the different infarcted areas before cell injections.

Morphometric Analysis

The capillary density was significantly higher in the areas of the anterolateral ventricular walls that received cell injections than in the previously infarcted posterior wall ($P<0.0001$) (Figure 1A). The median capillary density in the anterolateral wall was apparently similar to that in the septal wall. However, the broad dispersion of the septal wall data, which may have been due to fibrotic areas in regions close or adjacent to the ventricular walls, generated a statistically significant difference between these 2 groups.

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Figure 1. Number of capillaries per mm^2 in anterolateral, posterior, and septal walls of studied heart. A, Anti-factor VIII–associated antigen counterstained with hematoxylin. B, Anti-smooth muscle ▬-actin antigen counterstained with hematoxylin. C, Capillaries reacted with anti-factor VIII–associated antigen inside fibrotic areas only in anterolateral and posterior walls. (n=108 microscope fields for A; 96 microscope fields for B; and 40 microscopic fields for C.) Differences were statistically significant among all groups in pairwise comparisons ($P<0.05$, Newman-Keuls method) for A and B. Differences were significantly different ($P<0.05$) between anterolateral and posterior walls in Mann-Whitney rank-sum test for C.

The density of capillaries that contained smooth muscle α-actin–positive cells within their walls was also assessed (Figure 1B). The number of such vessels was higher in the anterolateral wall than in the septal and posterior walls ($P<0.0001$). Larger vessels identified by a continuous wall of smooth muscle α-actin–positive mural cells were not included in these analyses. The capillary density was significantly higher within fibrotic areas of the anterolateral wall than within fibrotic areas of the posterior wall ($P<0.0001$) (Figure 1C).

Histological Findings

The anterolateral wall showed irregular, pale regions of fibrotic tissue intercalated with dark regions of cardiac muscle arranged in roughly parallel, interspersed bands, perpendicular to the ventricular wall plane (Figure 2A). No abnormal cell organization, growth, or differentiation or signs of previous focal necrosis, inflammatory reactions, or tissue repair were found in the region that had received cell injections. Inside the fibrotic tissue, trichrome and picrosirius collagen staining disclosed regions with decreased collagen density, in which a rich vascular tree was present. The anterolateral wall also showed larger central vessels that ramified into smaller ones, parallel to the cardiomyocyte bundles (Figure 2B). In the anterolateral wall, the peripheral zone of fibrotic areas merged into the

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cardiomyocyte layer and lack_u well-defined limits, unlike the fibrotic areas observed in the posterior wall (Figure 2C). No fibrotic tissue was seen in the central area of the septal wall (Figure 2D).

Figure 2. Gomori trichrome stain of anterolateral (A, B), posterior (C), and septal (D) walls. Increased vascular tree is present in B. Original magnification is x40 in A, B, and D; x100 in C.

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Inflammatory cells were rare in the perivascular region: There were occasional isolated small groups of lymphocytes and, very rarely, granulocytes. At the interface between fibrotic tissue and cardiomyocyte bundles, 2 gradients merged: the decreasing blood vessel diameter and the increasing cardiomyocyte size. Very small cardiomyocytes were seen isolated in the fibrotic matrix adjacent to capillaries in the anterolateral wall, together with a progressively increasing number of fibroblastoid cells that were isolated or interspersed in small groups among the cardiomyocyte bundles.

Immunocytochemistry Findings

Immunocytochemical labeling of factor VIII–associated antigen identified a thin endothelial layer of blood vessels in the posterior, septal (Figure 3A), and anterolateral (Figure 3B) ventricular walls. In the anterolateral wall, neither factor VIII nor CD34 was found in the fibroblastoid cell population inside the fibrotic matrix. In the posterior ventricular wall and septum, smooth muscle α-actin was readily identified in blood vessel wall cells. This protein was present both in pericytes and in the smooth muscle cells of the thin vessel wall layer (Figure 3C) in the anterolateral wall. The vascular tree of the anterolateral wall showed intense labeling in the blood vessel walls, which had a marked hypertrophy of smooth muscle cells (Figure 3D). The same staining pattern was present in isolated cells located in the perivascular position and in the adjacent region among cardiomyocytes and fibrotic matrix (Figure 3E). Vimentin was present in the endothelial layer of the anterolateral wall, in the perivascular cells, and in cells adjacent to or in close contact with the cardiomyocytes (Figure 4A). These cells frequently formed an extensive network that permeated the fibrotic matrix and the

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interstitial space among cardiomyocytes (Figure 4B).

Figure 3. Immunocytochemical identification of factor VIII–associated antigen (A, B) and smooth muscle α-actin (C–E) in blood vessel walls of septal (A) and anterolateral (B–E) regions of studied heart, depicting increased vascular density (B) and hyperplasia of perivascular and mural cells (C–E). Ki67 reactivity was rarely present in perivascular cells of anterolateral wall (F). Original magnification x40 in A and B, x400 in C and D, and x1000 in E and F.

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Figure 4. Anterolateral wall that received cell injection therapy. A and B, Immunostaining for vimentin depicted positive reaction in vascular wall and in fibroblastoid interstitial cells. C and D, Immunostaining for desmin showed small groups of intensely reactive cells between blood vessels and cardiomyocytes (C) and small cells inside cardiomyocyte bundles with typical striated cytoskeleton (D). E and F, Immunostaining for troponin showed positive reaction in all mural cells of medium-sized blood vessel. Original magnification is x1000 in A and F; x400 in B–E.

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Desmin was identified in the same cell population. Desmin labeling was less intense in the vascular wall cells and isolated perivascular cells and was more intense in the cells adjacent to cardiomyocytes. On sections perpendicular to the main cardiomyocyte axis, thin desmin-positive fibrils were observed mainly in the submembrane region; on longitudinal sections, a typical transverse banded pattern of desmin was observed (Figure 4C and 4D). Among cardiomyocytes, some

of the small cells had strong, peripheral desmin-stained areas (Figure 4D).

In vascular and perivascular cells in the posterior wall and septum, troponin labeling was negative. In the anterolateral wall, troponin labeling was negative in capillary walls but was positive in the adjacent pericapillary pericytes and in cells migrating into the pericapillary matrix (Figure 4E). In larger vessels, troponin-positive cells were observed in the outer cell layers and adventitia, occasionally forming a continuous troponin-positive cell layer around the vessel (Figure 4F). Isolated cells or small groups of troponin-positive cells were found in the area between the fibrotic tissue and cardiomyocytes and inside the adjacent cardiomyocyte bundles. The intensity of labeling increased in the proximity of cardiomyocytes, where some small fibroblastoid cells disclosed a bright cytoplasm homogeneously labeled for troponin (Figure 5A). Occasionally, such cells had an increased volume, with troponin labeling restricted to the periphery; the central area was filled by a troponin-negative cytoskeleton similar to the desmin-stained areas in small cardiomyocytes. In mature cardiomyocytes, troponin-specific antibody labeled the peripheral filamentous and sarcomeric cytoskeleton.

Figure 5. Anterolateral wall that received cell injection therapy. A, Immunostaining for troponin depicted small cardiomyocyte-like cells with intense reaction in peripheral cell area. B–F, Immunostaining for sarcomeric actinin depicted reactivity in mural cells of blood vessel (B–E) and isolated cells among cardiomyocytes with actinin organization similar to that of sarcomeres (E, F). Original magnification is x400 in A and F; x1000 in B–E.

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Labeling of sarcomeric actinin was similar to that of troponin. However, both pericapillary pericytes and mural blood vessel cells in the anterolateral region were negative for sarcomeric actinin in blood vessels that were deeply embedded in the fibrotic scar matrix and that remained distant from cardiomyocyte bundles. The same cells located in vessels adjacent to or embedded between the cardiomyocyte bundles were positive for sarcomeric actinin, as were the isolated cells or small groups of fibroblastoid cells (Figures 5B and 5C). Some of these cells had increased in size and, in their central region, disclosed sarcomeric actinin that was already organized in the typical banded pattern of sarcomeres (Figures 5D and 5E). In this central region, isolated cells barely larger than pericytes

could be observed; only a few sarcomeres were present, suggesting that those isolated cells had acquired some cardiomyocyte characteristics (Figure 5F).

The Ki67 antibody, which identifies cells actively engaged in replication, reacted only rarely with endothelial cells in the posterior wall. In the anterolateral region, the Ki67 antibody also reacted with pericapillary pericytes and with isolated fibroblastoid cells in the surrounding fibrotic matrix (Figure 3F). The overall cell reactivity with Ki67 antibody was relatively low.

▶ Discussion

Accumulating evidence from both experimental animal studies[4-6] and human trials[7-9] indicates that ABMM cell therapy improves myocardial perfusion in patients with ischemic heart disease. At the same time, clinical stem cell therapy research is focusing more on safety than on efficacy. The present report describes the postmortem study of one patient who underwent transendocardial injection of ABMM cells. Accordingly, the major findings in this report pertain to the procedure's safety: No abnormal or disorganized tissue growth, no abnormal vascular growth, and no enhanced inflammatory reactions were observed. In addition, some intriguing histological and immunohistochemical findings were documented: (1) There was a higher capillary density in the cell-treated area than in nontreated areas of the heart. (2) A proliferation of smooth muscle ■-actin–positive pericytes and mural cells was noted. (3) The aforementioned cells expressed specific cardiomyocyte proteins.

In the postnatal period, new blood vessels form through either vasculogenesis or angiogenesis, in which proliferation of endothelial cells is followed by remodeling of the extracellular matrix and proliferation of blood wall cells.[13-15] Endothelial cells can result from bone marrow–derived progenitors (postnatal vasculogenesis) or from the migration and proliferation of endothelial cells from existing vessels (angiogenesis).[16] Mural cells such as pericytes and smooth muscle cells can be derived from bone marrow mesenchymal cells (stroma), myofibroblasts, and/or fibroblasts.[17] In the neoangiogenic process, pericytes are derived either from cells of adjacent tissues (mobilized by growth factors produced by endothelial cells) or from proliferation of adventitial and pericapillary pericytes and their distal gliding on the abluminal side of the growing blood vessel's basement membranes.[13] The alternative origination of pericytes from mesenchymal stem cells has been proposed and preliminarily confirmed in experimental models.[18] Pericytes may be essential to achieve a physiological angiogenic process with resultant durable blood vessels. In the present case, when compared with the noninjected regions, the cell-injected wall had marked hyperplasia of pericytes and mural cells. The observed hypertrophic pericytes displayed 2 characteristics: First,

although still located in the vascular wall, they expressed specific myocardial proteins and second, they were found in locations that suggested detachment, having migrated into the adjacent tissue and reached proximal cardiomyocytes that were either isolated or in small cell clumps. Closer to cardiomyocytes, the expression of myocardial proteins was enhanced, yielding brighter immunostaining throughout the whole cytoplasm. The significance of these findings remains to be established. However, within the posterior wall, none of the findings was seen, and small blood vessels could only rarely be found.

Notwithstanding the aforedescribed data, the present report has limitations that severely restrict our ability to make conclusions about the role of ABMM cells in myocardial regeneration. The findings could have occurred by chance. It is impossible to exclude the influence of a natural recovery process as the cause for the difference in vascular density between cell-treated and nontreated areas. Comparisons of capillary density among different sections of wall were based on specimens from a single patient. Moreover, this is an isolated, uncontrolled case involving late events after injection of unlabeled cells; it precluded the use of any imaging technique that could have helped to colocalize and identify the presence of stem cell direct descendants within the vessel wall or myocardium. Therefore, the significant difference in vascular density between cell-treated and nontreated areas cannot be extrapolated to a larger population of similar patients. However, the increased vascular density within the cell-injected anterolateral wall accompanied that wall's improvement in perfusion as assessed by SPECT, whereas all other walls remained unchanged.

▶ Conclusion

At 11-month follow-up evaluation, stem cell therapy was not associated with any adverse histological findings. Morphological and immunohistochemical analysis of the area that underwent ABMM cell implantation suggested that that area had more capillaries than nontreated areas and that ABMM cell therapy was associated with hyperplasia of pericytes, mural cells, and adventitia. Some of these cells had acquired cytoskeletal elements and contractile proteins (desmin, troponin, and sarcomeric actinin).

▶ Acknowledgments

assistance with statistical questions.

▶ Footnotes

*Drs Dohmann and Perin are coprincipal investigators. ▣

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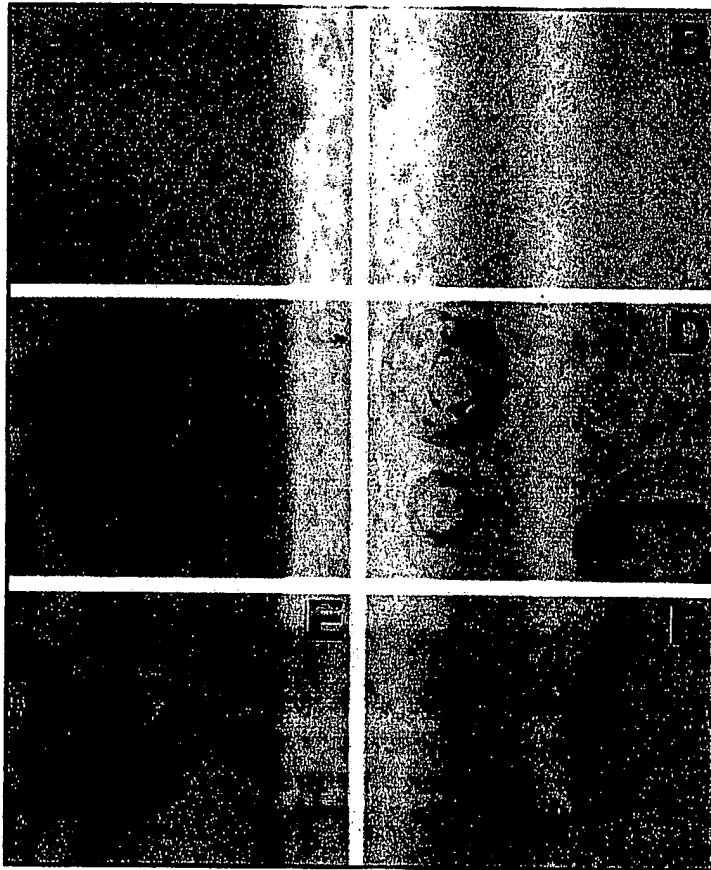
Figure 3. Immunocytochemical identification of factor VIII–associated antigen (A, B) and smooth muscle α-actin (C–E) in blood vessel walls of septal (A) and anterolateral (B–E) regions of studied heart, depicting increased vascular density (B) and hyperplasia of perivascular and mural cells (C–E).

EXHIBIT G

Strauer et al. 2005 publication in <u>Circulation</u> entitled,
"Regeneration of Human Infarcted Heart Muscle by
Intracoronary Autologous Bone Marrow Cell Transplantation
in Chronic Coronary Artery Disease" cited by Appellant
as Exhibit D in an Amendment dated November 21, 2005
in co-pending application Serial No. 09/974,456

Regeneration of Human Infarcted Heart Muscle by Intracoronary Autologous Bone Marrow Cell Transplantation in Chronic Coronary Artery Disease

The IACT Study

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OBJECTIVES	Stem cell therapy may be useful in chronic myocardial infarction (MI); this is conceivable, but not yet demonstrated in humans.
BACKGROUND	After acute MI, bone marrow-derived cells improve cardiac function.
METHODS	We treated 18 consecutive patients with chronic MI (5 months to 8.5 years old) by the intracoronary transplantation of autologous bone marrow mononuclear cells and compared them with a representative control group without cell therapy.
RESULTS	After three months, in the transplantation group, infarct size was reduced by 30% and global left ventricular ejection fraction (+15%) and infarction wall movement velocity (+57%) increased significantly, whereas in the control group no significant changes were observed in infarct size, left ventricular ejection fraction, or wall movement velocity of infarcted area. Percutaneous transluminal coronary angioplasty alone had no effect on left ventricular function. After bone marrow cell transplantation, there was an improvement of maximum oxygen uptake (VO_{2max}, +11%) and of regional ^{18}F-fluor-desoxy-glucose uptake into infarct tissue (+15%).
CONCLUSIONS	These results demonstrate that functional and metabolic regeneration of infarcted and chronically avital tissue can be realized in humans by bone marrow mononuclear cell transplantation. (J Am Coll Cardiol 2005;46:1651–8) © 2005 by the American College of Cardiology Foundation

Cardiac performance after myocardial infarction (MI) is compromised by ventricular remodeling, which represents a major cause of late infarct-related chronic heart failure and death (1,2). Although conventional drug therapy (e.g., with beta-receptor blockers and/or angiotensin-converting enzyme inhibitors) may delay remodeling, there is no basic

therapeutic regimen available for preventing or even reversing this process. By the use of interventional therapeutics (percutaneous transluminal coronary angioplasty [PTCA], stent), recanalization of the occluded infarct-related artery is possible, thereby improving or normalizing coronary blood flow. However, despite sufficient reperfusion of infarcted tissue, the viability of the infarcted myocardium cannot, or can only insufficiently, be improved in most of these patients (3). Therefore, catheter-based therapy of acute MI is useful for vascular recanalization, but the second and crucial step,

the regeneration of necrotic heart muscle, is not realized by this vascular procedure alone.

Experimental (4) and clinical (5,6) studies have shown recently for the first time that bone marrow mononuclear cells (BMCs) may regenerate damaged myocardium in acute MI in humans. Because the regenerative potential of bone marrow-derived cells ought also to be expected to exist in chronically ischemic heart disease as well (7–12), we have assembled in an ongoing clinical investigation 18 patients with chronic MI to prove this new therapeutic possibility.

METHODS

Study population. All 18 patients (49 ± 11 years) were men and were recruited consecutively from January 2003 until March 2004. They had had transmural MI 27 ± 31 months before, at which point all infarcts had been treated acutely by PTCA and/or stent implantation (Table 1, Fig. 1).

The inclusion criteria were age <70 years, one-vessel disease with an open infarct-related artery at the time of stem cell therapy, sinus rhythm, a clear-cut demarcation of the ventriculographic infarct area, and no coronary bypass surgery. General exclusion criteria were severe comorbidity and alcohol or drug dependency. Although chronically infarcted myocardium usually does not regenerate sponta-

Abbreviations and Acronyms

BMC = bone marrow mononuclear cell
CPK = creatine phosphokinase
ECG = electrocardiogram
LV = left ventricular
MI = myocardial infarction
PET = positron emission tomography
PTCA = percutaneous transluminal coronary
        angioplasty
Tx group = transplantation group

neously, for comparison a control group, parallel to the recruitment of the stem cell transplantation group (Tx group), was recruited and analyzed, meeting the same inclusion criteria as the stem-cell group. The recruitment of patients was performed according to a randomization procedure in which all patients of the entire chronic infarction group were distributed to the treatment group, where they agreed with all the therapeutic regimen. Alternatively, all patients of the chronic infarction group who refused the therapeutic regimen (bone marrow puncture and aspiration, intracoronary cell transplantation, and another cardiac catheterization) were allocated to the control group. All medications with angiotensin-converting enzyme inhibitors and with beta receptor blockers were maintained constant during the study period.

The cell-treated patients had stable ventricular dynamics for infarct size, ejection fraction, and wall movement velocity of infarcted area at least 9 ± 6 months before cell transplantation. Infarct size at the time of cell therapy showed an amount of 27 ± 8% of the circumference of the left ventricle (LV), determined by ventriculography.

Preparation of BMCs. One day before cell therapy, bone marrow was taken (80 ml from the iliac crest) and mono-

nuclear cells were isolated and identified including CD34-positive cells, AC133-positive cells and CD45/CD14 negative cells (6). The cells were isolated under good manufacturing practice conditions by Ficoll density separation on Lymphocyte Separation Medium (Bio Whittaker, Walkersville, Maryland), before the residual erythrocytes were lysed with H_2O. For overnight cultivation, 1×10^6 BMCs/ml were placed in Teflon bags (Vuelife, Cell Genix, Gaithersburg, Maryland) and cultivated in X-Vivo 15 Medium (Bio Whittaker) supplemented with 2% heat-inactivated autologous plasma. The next day, BMCs were harvested and washed three times with heparinized saline before final resuspension in heparinized saline. Viability was 93 ± 3%. Heparinization and filtration (cell strainer, FALCON) was carried out to prevent cell clotting and microembolization during intracoronary transplantation. These cells were used for therapy. All microbiologic tests of the clinically used cell preparations proved negative. All patients received extensive information about the procedure, which was approved by the ethical committee of our university, and all gave written informed consent.

Administration of BMCs. Following assessment of baseline examinations (coronary angiography, left ventriculography, spiroergometry, ^{99m}Tc-tetrofosmin single-photon emission computed tomography (SPECT) and ^{18}F-fluor-deoxy-glucose (^{18}F-FDG) positron emission tomography (PET), cell transplantation was performed via the intracoronary administration route (6,13) using four to six fractional infusions parallel to balloon inflation over 2 to 4 min of 3 to 5 ml of cell suspension, each containing 15 to 22 × 10^6 mononuclear cells. All cells were infused directly into the infarcted zone through the infarct-related artery via an angioplasty balloon catheter, which was inflated at a low pressure (2 to 4 atm) and was located within

Table 1. Demographic Data of Intracoronary Bone Marrow Stem Cell Transplantation Group and Control Group

Characteristics	Tx Group	Control Group	p
No. of patients	18	18	
Age, yrs	49 ± 11	52 ± 10	NS
Transmural myocardial infarction, months before Tx	27 ± 31	30 ± 34	NS
Coronary angiography			
LAD/LCX/RCA as affected vessel	16/0/2	10/3/5	
No. of patients with stent implantation	16	17	NS
Risk factors			
Diabetes mellitus, %	16	11	NS
Positive family history, %	44	33	NS
Smoker and ex-smoker, %	67	56	NS
Hyperlipoproteinemia, %	89	94	NS
Medication			
Beta-blocker, %	94	89	NS
Angiotensin-converting enzyme inhibitor, %	94	89	NS
Statin, %	94	100	NS
Laboratory parameters			
CPK, U/l	1,504 ± 979	1,489 ± 952	NS
Bone marrow mononuclear cells, n (10^6×)	90		

Values are mean ± SD or number of patients.
CPK = creatine phosphokinase; LAD = left anterior descending coronary artery; LCX = left circumflex coronary artery; RCA = right coronary artery; Tx = intracoronary bone marrow stem cell transplantation.

Figure 1. Diagrammatic representation of the algorithm of intracoronary stem cell therapy (Tx) in chronic ischemic heart disease after myocardial infarction. The infarcts occurred 27 ± 31 months before Tx. All infarct patients were treated with percutaneous transluminal coronary angioplasty (PTCA) or with stent implantation. 9 ± 6 months before (investigation 1) coronary angiography (including quantitative left ventriuclography) was performed. If re-stenosis was present, re-PTCA was made. Investigation 2 embraces all patients for the evaluation of coronary morphology after PTCA/stent. Only patients with an open infarct-related artery were included in both groups. Patients who agreed to Tx received within 10 days after investigation 2 bone marrow punctures and Tx by the intracoronary administration route and had altogether five invasive investigations, including two for therapeutic reasons (nos. 0 and 1). Patients who were not eligible for Tx (disagreement with bone marrow puncture and with subsequent Tx) served as a control group. Investigation 3 represents all follow-up measurements 3 months after Tx (Tx patients) or after investigation 2 for control group patients.

the previously stented coronary segments. This prevented backflow of cells and produced stop flow beyond the site of balloon inflation to facilitate high-pressure infiltration of cells into the infarcted zone. Prolonged contact time for cellular migration was also enabled. Three months after catheter-guided cell transplantation, all functional tests were repeated, including coronary angiography and left ventriculography. There were no procedural or cell-induced complications, and there were no side effects in any patient.

Spiroergometry. Aerobic exercise capacity was examined before (<10 days) intracoronary cell transplantation and three months later during follow-up. All patients (n = 18) were subjected to initial bicycle spiroergometry to assess their functional fitness and to determine the limit of safe intensity of exercise. We chose a protocol with an intensified workload up to the symptom-limited maximum (basic load of 50 W, intensification at 25 W, 2-min duration of each workload step). We determined the anaerobic threshold for prescribing a suitable load intensity. During the whole spiroergometry, monitoring by a 12-lead electrocardiogram (ECG) was carried out. The exercise capacity was assessed on the basis of maximum load levels expressed in watts (W_{max}) and maximum peak oxygen uptake (Vo_{2max}).

Coronary angiography and left ventriculography. Coronary angiography and biplane left ventriculography were performed 9 ± 6 months before cell transplantation and also a second time, within 10 days, immediately before cell therapy. The therapeutic follow-up was three months after the treatment. Thus, stable baseline conditions were documented (coronary vessel involvement, ventricular function, and geometry). Cardiac function was evaluated by left

ventricular (LV) ejection fraction and by auxotonic myocardial contractility index, evaluated by the wall movement velocity of the infarcted area. The infarct size was calculated according to the method of Sheehan (14) by plotting five axes perpendicular to the long axis of the heart in the main akinetic or dyskinetic segment of the ventricular wall. Systolic and diastolic lengths were then measured by two independent observers, and the mean difference was divided by the systolic duration in seconds.

Quantification of coronary stenosis (restenosis). Cinecoronarangiograms were obtained during stem cell transplantation and at three months thereafter according to standard acquisition guidelines. The angiograms were evaluated by two independent observers and quantitative analysis was performed (15). Standard morphologic criteria were used to characterize the complexity of baseline lesions. The user-defined reference diameter proximal to the stenosis and the minimal luminal diameter within the culprit of the stenosis were used to calculate the percentage of stenosis. A value of 0 mm was assigned for the minimal luminal diameter in case of total occlusion at baseline or follow-up. Restenosis was defined as ≥50% stenosis of the initial target lesion at follow-up. Calculations of restenosis were performed in both groups, with and without stem cell therapy, in the same way, thus enabling evaluation the differential effects of PTCA-guided cell therapy and of PTCA effects alone.

Ventricular function after PTCA in the control group. For the evaluation of a potential effect on the PTCA intervention itself on LV function, all patients in the control group were analyzed with regard to infarct size, ejection fraction, and infarction wall movement velocity.

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Table 2. Single Values of Intracoronary Bone Marrow Stem Cell Transplantation Group

Patient Number	Area of Infarction, %*			LV Ejection Fraction, %*			Infarction Wall Movement Velocity, cm/s*		
	Investigation 1 9 ± 6 Mo Before Tx	Investigation 2 <10 Days Before Tx	Investigation 3 3 Mo After Tx	Investigation 1 9 ± 6 Mo Before Tx	Investigation 2 <10 Days Before Tx	Investigation 3 3 Mo After Tx	Investigation 1 9 ± 6 Mo Before Tx	Investigation 2 <10 Days Before Tx	Investigation 3 3 Mo After Tx
1	26	26	22	56	55	60	0.88	0.77	0.82
2	28	29	26	45	43	49	2.06	1.88	2.13
3	16	16	5	64	65	71	1.45	1.50	2.10
4	27	25	14	48	50	65	1.20	1.25	2.88
5	16	14	11	66	69	71	2.25	2.77	3.75
6	16	13	6	64	66	73	1.50	1.77	2.55
7	15	18	11	57	55	63	2.78	2.65	3.13
8	28	28	20	43	44	49	3.15	3.25	4.25
9	27	27	11	46	46	64	1.61	1.65	3.30
10	20	17	14	56	58	62	2.21	2.45	3.13
11	28	25	17	42	38	52	1.91	1.88	3.00
12	33	28	21	44	47	54	2.28	2.62	3.50
13	39	37	27	50	51	59	1.25	2.50	4.90
14	29	33	27	62	62	61	1.20	1.33	2.70
15	37	37	31	48	43	53	1.83	1.56	2.50
16	29	29	24	53	54	58	1.25	1.06	3.06
17		41	35		48	55		1.66	3.00
18		35	25		45	53		0.94	1.94
Mean	26	27	19	53	52	60	1.80	1.86	2.92
SD	7	8	9	8	9	7	0.63	0.70	0.91

*Calculated from left ventriculography.
LV = left ventricular; Mo = Months; other abbreviations as in Table 1.

Nuclear cardiologic investigations (PET and SPECT). ^{18}F-FDG-positron emission tomography (^{18}F-FDG PET) was performed with a Scanditronix SCX 4096 WB-Scanner (FWHM = 6 mm transaxial, axial field of view = 4.6 cm). Patients received an oral glucose load of 1 g/kg body weight 80 ± 30 min before the intravenous application of ^{18}F-FDG (380 ± 60 MBq). The ^{18}F-FDG was administered at the time of decrease of blood glucose level <130 mg/dl. An initial transmission scan was obtained using a ^{68}Ga-filled pin source to correct the subsequent emission scans for attenuation. The data acquisition was started 45 min after administration of FDG. Image data were recorded with a 256 × 256 matrix in 3 consecutive bed positions over 15 min per position. The data were reconstructed back-projected with a Hanning filter (5 mm).

[99m]Tc-tetrofosmin SPECT. Sixty minutes after intravenous injection of 600 ± 140 MBq of the perfusion-marker ^{99m}Tc-tetrofosmin under a "rest" condition, the images were obtained using a SPECT scanner with double-head detector (PRISM 2000, Marconi/Phillips), a low-energy, high-resolution collimator, and a 128 × 128 matrix. Image data were collected over 360° at 3° every 30 s. The images were reconstructed backprojected with a low-pass filter (order 12, cutoff 0.2).

PET and SPECT evaluation. Normalized values for FDG uptake and perfusion were calculated by comparing regional with maximum tracer uptake on the reconstructed images. We performed a regional analysis of glucose metabolism and perfusion using a set of standardized, individually adjusted circular regions of interest (diameter 18.06 mm, surface 256 mm^2). The reconstructed metabolic and perfusion images were realigned for each patient (MPI-Tool, version 3.0; Advanced Tomo Vision, Erftstadt, Germany) and were resliced according to cardiac axis (short-axis and horizontal and vertical long-axis views). The regions were positioned immediately neighboring, with no overlap, according to an overlay of the co-registrated metabolic and perfusion images. The regions covered the infarct lesion as well as normal myocardium. In this way, we generated templates of regions for each patient, which could be used for the evaluation of metabolism and perfusion, before and after BMC transplantation without further modification. According to Segall et al. (16), regions with a normalized FDG uptake <50% were rated as transmural scar and regions with an uptake of 50% to 60% as non-transmural scar.

Further analysis was restricted to regions with FDG uptake <60% in the PET scans, pursuant to our intention to focus on the effects of BMC transplantation on scar tissue.

Safety parameters. To assess any inflammatory response and myocardial reaction after cell therapy, white blood cell count, the serum levels of C-reactive protein (CRP) and of creatine phosphokinase (CPK) were determined immediately before as well as after treatment. Additional analysis was done directly after transplantation and three months later: ECG at rest, 24-h Holter ECG, and echocardiography.

Statistical analysis. All data are presented as mean ± SD. Statistical significance was accepted when p < 0.05. Intra-individual comparison of variables of investigation 1 (9 ± 6 months before cell transplantation for Tx group, 9 ± 5 months before investigation 2 for control patients) and investigation 2 (<10 days before cell transplantation for Tx group, no transplantation for control patients) and of variables of investigation 2 and follow-up investigation 3 (3 months after cell therapy for Tx group, 8 ± 5 months after investigation 2 for control patients) was performed using Wilcoxon rank-sum test. The missing values (Table 2) were omitted and not calculated for statistical analysis. The p values (by analysis of variance) have been given for LV ejection fraction, area of infarction, and infarction wall movement velocity. Statistical analysis was performed with SPSS-Windows 10.1 software.

RESULTS

Three months after intracoronary cell therapy, the infarct size was reduced by 30%, whereas the global LV ejection fraction increased by 15% and regional infarct wall movement velocity by 57% (Tables 2 and 3). In parallel, the clinical performance improved (Table 4), as evidenced by a higher work load demonstrated by a 11% increase in maximum oxygen uptake (Vo_{2max}). SPECT investigation presented enhanced tetrofosmin uptake in the infarcted zone by 5%, and PET examination showed enhanced glucose uptake in the infarcted zone by 15%, demonstrating regeneration of formerly avital, chronically infarcted heart muscle (Fig. 2). An unchanged or even impaired LV function was not observed in any patient.

In the control group (18 patients with chronic MI, but without stem cell therapy) no significant changes were observed in infarct size, LV ejection fraction, or wall

Table 3. Cardiac Parameters in the Transplantation Group and in Control Group at the Three Investigation Time Points

	Area of Infarction, %			LV Ejection Fraction, %			Infarction Wall Movement Velocity, cm/s		
	Control Group	Tx Group	p Value*	Control Group	Tx Group	p Value*	Control Group	Tx Group	p Value*
Investigation 1	25 ± 9	26 ± 7	0.99	53 ± 10	53 ± 8	0.87	1.95 ± 0.66	1.80 ± 0.63	0.57
Investigation 2	27 ± 9	27 ± 8	0.83	51 ± 10	52 ± 9	1.00	1.88 ± 0.76	1.86 ± 0.70	0.94
Investigation 3	26 ± 9	19 ± 9	0.02	52 ± 10	60 ± 7	0.02	1.91 ± 0.79	2.92 ± 0.91	0.001

*Analysis of variance.
Abbreviations as in Table 1.

1656   Strauer et al.
Regeneration of Infarction by BMCs in Chronic CAD

JACC Vol. 46, No. 9, 2005
November 1, 2005:1651-8

Table 4. Positron Emission Tomography and Spiroergometry Before and After Stem Cell Therapy in Chronically Infarcted Myocardium

	^{18}F-FDG-Positron Emission Tomography		Vo_{2max}, Spiroergometry	
	FDG Uptake, %	Difference in %	ml/min	Difference in %
Investigation 1	none		none	
Investigation 2	43.8 ± 8.0	> +15	1,602 ± 533	> +11
Investigation 3	50.5 ± 11.6		1,776 ± 523	
p (Wilcoxon test)	0.012		0.0001	

^{18}F-FDG = ^{18}F-fluor-deoxy-glucose; Vo_{2max} = maximum oxygen uptake.

movement velocity of the infarcted area (Figs. 3A to 3C). Electrocardiogram at rest and on exercise and 24-h Holter ECG revealed no rhythm disturbances at any time point. Only 1 patient (from 18 cell-treated patients, 6%) developed relevant restenosis due to quantitative angiographic criteria. The restenosis could be treated adequately by stent implantation. The other 17 patients showed good patency rates without restenosis after PCI and cell transplantation. They also revealed no alterations in LV function 8 ± 5 months after PTCA.

There was no inflammatory response or myocardial reaction (white blood cell count, CRP, CPK) after cell therapy, despite a moderate increase in CRP (before cell transplantation 0.58 ± 0.48 mg/dl, after cell transplantation 1.07 ± 0.73 U/l, p = 0.002), which is usual after bone marrow puncture and/or cardiac catheterization.

DISCUSSION

The results of these investigations demonstrate, for the first time, that the intracoronary transplantation of autologous bone marrow mononuclear cells may reduce infarct size and improve LV function as well as myocardial glucose uptake in chronic ischemic heart disease attributable to chronic MI (5 months to 8.5 years old). Infarct size decreased in all patients and cardiac performance (ejection fraction, wall movement velocity of infarcted area, maximum oxygen uptake, and exercise tolerance) and myocardial metabolism (FDG-PET) improved, all being between 11% and 57%. Furthermore, it is noteworthy that there were no complications immediately or three months after cell transplantation, especially that there was no cardiac arrhythmia and no signs of cardiac or systemic inflammation were present.

The effects of stem cell transplantation on infarct size, cardiac function, and contractility demonstrate significant improvement of these three parameters in the therapy group (before and after stem cell therapy) as well as in the comparison between the stem cell therapy group and the control group, thus giving evidence for a beneficial therapeutic effect of stem cell therapy on cardiac performance in chronic MI.

Patients in both the stem-cell group and the control group were recruited in parallel to each other and consecutively between January 2003 and March 2004. They all (n = 36) fulfilled the same inclusion criteria. Thus, representative patient characteristics were present for the stem cell group (n = 18) and the control group (n = 18) as well as in comparing both of them. Moreover, two subsequent investigations before stem cell transplantation have been performed for each patient: investigation 1 and 2 demonstrated the stability of LV dynamics before cell therapy (9 months respectively 10 days before transplantation) and investigation 3 compared the effects of stem cell therapy with the control group. The stable hemodynamics during the preceding 9 ± 6 months before stem-cell therapy and the stable hemodynamics within the control group at all three points of investigation underline the significant alterations of the left ventriculography-derived parameters investigated after stem cell transpantation.

The regenerative potential of bone-marrow–derived stem cells may be explained by any of four mechanisms: 1) direct cell differentiation from mononuclear cells to cardiac myocytes (17), 2) cytokine-induced growing and increase of residual viable myocytes, especially within the border zone of the infarcted area (18), 3) stimulation of intrinsic myocardial stem cells (endogenous stem cells) (19,20), and 4)

Lateral

Apex   **Base**

Septal

Figure 2. Representative illustration of ^{18}F-FDG-positron emission tomography (PET) before (above) and 3 months after (below) cell therapy in the transversal (left) and longitudinal (right projection) in a 30-year-old male patient with an 8-month-old anteroapical infarction. Note the restoration of glucose uptake (below) within the infarcted area of the formerly completely avital anteroapical myocardium.

Figure 3. Illustration of the mean values of (A) area of infarction, (B) ejection fraction, and (C) infarction wall movement velocity, determined by quantitative left ventriculography in both groups (control group vs. transplantation [Tx] group) at the point of time: investigations 1, 2, and 3. Comparision of both groups with chronically infarcted myocardium (conrol group vs. Tx group), n = 18 patients. Investigation 1 was 9 ± 6 months before cell transplantation (controls: 9 ± 5 months before percutaneous transluminal coronary angioplasty [PTCA]); investigation 2 within 10 days before cell tranplantation (controls: at the time point of PTCA) and investigation 3 was three months after cell transplantation (controls: 8 ± 5 months after PTCA). Note the significant decrease of infarct size and the increase in ejection fraction and in contractility (infarction wall movement velocity) 3 months after cell therapy in comparison with the control group. *p = not significant (investigation 2 vs. investigation 3); #p = 0.001 (investigation 2 vs. investigation 3).

induction of cell fusion between transplanted bone marrow cells and resident myocytes (21–24).

Transdifferentiation has been described by previous investigators (4); however, it has been questioned by recent experimental studies (25). The influence of cytokines has shown to restore coronary blood vessels and muscle cells after experimental myocardial infarction. This regeneration of blood vessels and muscle cells is most pronounced in the border zone of ischemic and/or infarcted tissue (26), demonstrating an enhancement of mitotic cells and cell cycles up four-fold, when compared to areas remote from the necrotic myocardium. Moreover, mononuclear bone marrow stem cells contain a lot of cytokines (VEGF, insulin-like growth factor, platelet-derived growth factor, and so on), thereby stimulating residual normal myocytes for regeneration and proliferation and instrinsic myocardial stem cells (endogenous stem cells) for cell regeneration and for cell fusion (27–31).

Mitotic indexes are three to four times more frequent within the border zone of myocardial necrosis when compared with non-injured heart muscle (26). Moreover, 20% to 40% of intracoronarily transplanted bone-marrow-derived stem cells may be accumulated within the border zone of MI. There were no signs of apparent microcirculation disturbances because all patients had Thrombolysis In Myocardial Infarction flow grade 3. Thus, it is conceivable that in MI the border zone represents the optimum "niche" for exogenously transplanted stem cells, stimulating mitosis rates and heart muscle regeneration, preferably originating in and expanding from these areas. Cell fusion may also contribute to heart muscle regeneration, which takes its origin from the border zone, expanding gradually to the necrotic core of the infarcted area.

Our study cannot determine which cell-biologic and molecular mechanims are responsible for heart muscle repair or which of the studied factors may play the predominant role. However, the final functional outcome of this cell therapy demonstrates three main target effects: improvement in muscle function (pumping ability and contractility), myocardial perfusion (SPECT), and myocardial glucose metabolism (PET), thus giving evidence that heart muscle repair must have taken place by this intracoronary bone marrow cell transplantation procedure.

The clinical significance of this novel therapeutic approach may embrace a large number of patients with chronic coronary artery disease, preferably after previous or long-standing MI. It is conceivable that remodeling after infarction may be ameliorated or even stopped by this procedure. Thus, cell therapy may represent a new option of basic and causal therapy in chronic infarcted myocardium. It is an open question whether variations of the amount and kind of bone marrow cells, the administration technique, and the transplantation procedure itself, by enhanced environment and improvement of the angiogenic micromilieu, can further improve the milieu-dependent differentiation or regeneration of bone marrow cells in chronic infarcted heart disease. Therefore, our clinical results represent a stable basis to proceed to the next necessary step: to a larger prospective randomized study.

Reprint requests and correspondence: Dr. Bodo E. Strauer, Head of Department of Medicine, Division of Cardiology, Pneumology, and Angiology, Heinrich-Heine-University, Moorenstr. 5, 40225 Düsseldorf, Germany. E-mail: strauer@med.uni-duesseldorf.de.

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EXHIBIT H

Final Rejection dated September 22, 2006,
page 22, first paragraph, issued in co-pending
application Serial No. 09/836,750

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/836,750	04/17/2001	James P. Elia	1000-10-C01	7239

7590   09/22/2006

Gerald K. White
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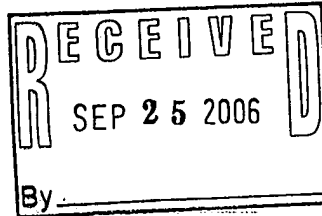
EXAMINER
KEMMERER, ELIZABETH

ART UNIT	PAPER NUMBER
1646	

DATE MAILED: 09/22/2006

Please find below and/or attached an Office communication concerning this application or proceeding.

	Application No.	Applicant(s)
Office Action Summary	09/836,750	ELIA, JAMES P.
	Examiner	Art Unit	
	Elizabeth C. Kemmerer, Ph.D.	1646	

-- *The MAILING DATE of this communication appears on the cover sheet with the correspondence address* --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE <u>3</u> MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on <u>26 June 2006</u>.

2a)☒ This action is **FINAL**.   2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) <u>6-236,238-253 and 256-287</u> is/are pending in the application.

     4a) Of the above claim(s) <u>6-235 and 240-242</u> is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) <u>236,238,239,243-253 and 257-287</u> is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

     Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

     Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

     a)☐ All   b)☐ Some * c)☐ None of:

       1.☐ Certified copies of the priority documents have been received.

       2.☐ Certified copies of the priority documents have been received in Application No. _____.

       3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

     * See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1)☒ Notice of References Cited (PTO-892)

2)☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)

3)☐ Information Disclosure Statement(s) (PTO/SB/08)
     Paper No(s)/Mail Date _____.

4)☐ Interview Summary (PTO-413)
     Paper No(s)/Mail Date. _____.

5)☐ Notice of Informal Patent Application

6)☐ Other: _____.

DETAILED ACTION

Status of Application, Amendments, And/Or Claims

The amendment received 26 June 2006 has been entered in full. Claims 1-5, 237, and 254-256 are canceled. Claims 6-235 and 240-242 remain withdrawn from consideration as being directed to a non-elected invention. Claims 236, 238, 239, 243-253, and 257-287 are under examination.

The fourth supplemental declaration of Dr. Heuser under 37 CFR 1.132 and third supplemental declaration of Dr. Lorincz under 37 CFR 1.132 submitted with the response have been entered. A copy of the third supplemental declaration of Dr. Heuser under 37 CFR 1.132 has also been received.

The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

As an initial matter, it is noted that Applicant comments upon alleged procedural errors. The record has been reviewed and no errors in procedure have been noted. Therefore, these comments will no longer be addressed further.

35 U.S.C. § 112, First Paragraph, New Matter

Claims 248, 249, 252, and 274-279 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. This is a new matter

second piece of evidence, Merck has to do with cancer and does not appear to be

relevant. The third piece of evidence, NIH report, lists results of a web search for

"nonspecific growth factor" and also appears to be irrelevant to the issue at hand. The

last piece of evidence, Exhibit III in the after final amendment, reviews traditional use of

cells for cancers and immunotherapy, and newer uses as gene therapy vehicles. None

of these treatments involve the systemic administration of cells to repair a distant organ.

Applicant argues that administration of cells is old in the art. This point is

conceded.

Applicant argues that the examiner's statement regarding Deb et al., wherein it

was acknowledged that cells administered intravenously could migrate to the art, should

end all speculation regarding enablement of the claimed invention. This has been fully

considered but is not found to be persuasive because Deb et al. do not demonstrate

that cells can migrate to the heart in sufficient quantities to repair any defects. Deb

discloses that only $0.23 \pm 0.06\%$ of the cardiomyocytes were from the transplanted

cells. Such numbers of cells are greatly insufficient to achieve the effects required by

the claims. As evidence of this, Strauer 2002 administered 6 to 7 fractional high-

pressure infusions of 2 to 3 mL cell suspension, each of which contained 1.5 to 4 X 10^6

mononuclear cells directly to the infarct site in order to achieve their effects. In fact,

Strauer 2002 specifically points to shortcomings of intravenous administration at p.

1917. The evidence as a whole indicates that intravenous administration of cells to

repair a dead or damaged portion of a heart has not yet been achieved due to the

obstacles involved with getting sufficient numbers of cells to the dead/damaged site and

COPIES OF ITEMS 1-31

Cited in

EVIDENCE APPENDIX

EVIDENCE APPENDIX

ITEM NO. 1

Declaration of Dr. C. Gene Wheeler cited by Appellant as an Exhibit in the Amendment filed February 15, 2001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: James P. Elia)
)
SERIAL NO.: 09/064,000)   EXAMINER: Nicholas D. Lucchesi
)
FILED: April 21, 1998)
)   GROUP ART UNIT: 3732
FOR: METHOD AND APPARATUS)
    FOR INSTALLATION OF)
    DENTAL IMPLANT)

DECLARATION OF C. GENE WHEELER, M.D., F.A.C.S.

I, C. Gene Wheeler, declare as follows:

1.   I reside at 6342 E. Hillcrest Boulevard, Scottsdale, Arizona 85251.

2.   My Curriculum Vitae is attached hereto as Exhibit A.

3.   I have read and understood the disclosures at column 14, lines 4-61 and column 21, lines 1-26 of United States Patent Number 5,397,235 (hereinafter " '235 patent") entitled "Method for Installation of Dental Implant," and granted to James P. Elia on March 14, 1995. A copy of such disclosures is attached hereto as Exhibit D. I understand that the same disclosures are contained in above patent application Serial No. 09/064,000.

4.   I note that the disclosures mentioned in above Paragraph 3 relate to a method for forming a bud and then for forming soft tissue. Such methods involve placing a growth factor at a desired site of a body with use of techniques including resorbable and non-resorbable carriers, gels, time-

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release capsules, and granules. In addition, the growth factor may be placed in the body orally, systemically, by injection, through the respiratory tract, by making an incision in the body and then inserting the growth factor. I note further that the growth factor and/or carrier may be activated by tissue pH, enzymes, ultrasound, electricity, heat, or in vivo chemicals.

5. It is well known and established in the medical arts that buds are a primordium or, in other words, a rudiment or commencement of an organ. The process of organ formation includes the differential development of cells to form an organ primordium with the resulting formation of soft tissue. Such process of development is called organogenesis. It is also well known and established in the medical arts that the term "soft tissue" includes blood vessels.

    In making the above statement in this Paragraph, I am aware of the definitions attached hereto as Exhibit B. Terms included in the above-mentioned definitions are: bud, primordium; organogenesis, and organ. I am also aware of and have considered the definition of "growth factor" as contained in Column 14 of the aforesaid '235 patent.

6. The materials included in attached Exhibit C evidence that the placement of growth factors in the body of a human results in the formation of a bud with subsequent growth into soft tissue. These materials report work performed by reputable, skilled scientists and reputable organizations in the medical arts. Consequently, I believe that these reports would be recognized as clearly valid by one of ordinary skill in the medical arts because they report the results of scientific tests conducted by competent, disinterested third parties with use of proper scientific controls.

7. Based upon the materials included in above Paragraphs 4, 5, and 6, it is my opinion that the process of placing a growth factor at a desired site of a human body will produce a bud that will predictably subsequently grow into soft tissue, as described in the '235 patent, using the techniques identified in above Paragraph 4. My further opinion is that when the techniques and angiogenic growth factors described and disclosed in the Elia patent application are used to place such growth factors in a human host, such placement would result in the formation of soft tissue, e.g., blood vessels. My opinion is in accord with the results obtained by the Isner patent (Exhibit C-6) which employed the same angiogenic growth factors and carrier/technique described and disclosed in the Elia patent application.

8. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 2/11/01

C. Gene Wheeler

C:\MYDOCUMENTS\CLIENTS\ELIA\WHEELERDECLARATION.DOC

3

EXHIBIT A

CURRICULUM VITAE

C. GENE WHEELER, M.D.,F.A.C.S.
6342 E. Hillcrest Blvd.
Scottsdale, Arizona 85251
Home Phone: (480) 941-8345

CURRICULUM VITAE

Clarence Gene Wheeler

Born:

May 23, 1930
U.S. Citizen

Education:

Bachelor of Science, Wheaton College, Wheaton, Illinois, August
     1951 with highest honors
Baylor University College of Medicine, May 1955, M.D. with
     highest honors
Surgical Internship: Massachusetts General Hospital,Boston,
     Massachusetts 1955-1956
Surgical Residency: Massachusetts General Hospital, Boston,
     Massachusetts 1956-1962
Cardiovascular Fellowship: Methodist Hospital, Houston, Texas:
     Dr. DeBakey and Dr. Cooley, September
     1960 - June 1961

Academic:

Prior: Clinical Associate Professor of Surgery, Southwestern
     Medical School, Dallas, Texas
     Clinical Associate Professor of Surgery, University of
     Arizona College of Medicine, Phoenix Campus

Boards:

American Board of Surgery 1963

Hospital Affiliations:

Attending Surgeon, Baylor University Medical Center, Dallas,
     Texas, 1962 to June 1990 (resigned)
Chief, Vascular Surgery, Carl T. Hayden VA Medical Center,
Phoenix, Arizona 1990 - 1999

Organizations:

American College of Surgeons 1966
The Cooley Cardiovascular Society
The DeBakey Cardiovascular Society
The International Cardiovascular Society
Texas Surgical Society
Phoenix Vascular Group - Co-founder 1991

Other Interests:

Fishing, Skiing,Tennis, Security and Real Estate Investment,
Missionary Surgery (Mexico, Central America, India)

Community Interests:

The Episcopal Foundation, Terrell Branch, Terrell, Texas
Gideon International, Central Phoenix Branch, Phoenix, Arizona
Former Member, Vestry Board of the Good Shepherd Episcopal
     Church, Terrell, Texas
Former Member, Vestry of St. Matthew's Cathedral, Dallas, Texas

C. GENE WHEELER, M.D.,F.A.C.S.
CURRICULUM VITAE

Community Interests
Continued:

	Former Member, Deacon Board of the First Baptist Church, Dallas, Texas
	Former Trustee of Dallas Theological Seminary, Dallas, Texas
	Former member, Vestry Board, Christ Church of Ascension - Episcopal, Paradise Valley, Arizona
Teaching Assignments:	Director of Vascular II Surgical Service, Baylor University Medical Center, Dallas, Texas 1970-1985
	Director of Vascular Training Fellowship, Baylor University Medical School, 1968-1985
	Founder of Visiting Vascular Lectureship, Baylor University Medical Center, Dallas, Texas 1978
	Faculty Member, Phoenix Integrated Surgical Residency Program, 1990
	Co-Director, Founder - National PACT Training Program, 1995
Administrative Experience:	Senior Partner and Manager, Surgical Associates of Dallas, Dallas, Texas, 1970-1985 - Surgical Practice and Financial Management Affairs
	Member of Texas Medical Foundation Regional Quality Assurance Committee, Dallas, Texas, 1988-1989
	Member, Infection Control Committee
	Member, Quality Assurance Committee
	Member, ICU-CCU Committee
	Member, Hospital By-Laws Committee
	Member, Pharmacy and Therapeutics Committee
	Member, Institutional Review Board, Carl T. Hayden VAMC
Investigations in Progress:	Chief Investigator: C.G. Wheeler, M.D.
	1. Safe Carotid Endarterectomy - Ongoing Quality Care Series, 1990
	2. Below Knee PTFE Reconstruction with AV Fistula in Diabetic Patients - IMPRA, Inc., 1993
	3. Operation Desert Foot - Amputation Prevention Program, and Statistical Analysis, 1991 to present
	4. TOPAZ Trial (Thrombolysis in Acute Arterial Occlusion) - Abbott Laboratories, 1993
	5. PURPOSE Trial, Abbott Laboratories, 1997
Presentations:	Presiding Officer, Phoenix Vascular Group, Vascular Lectureship, March 1991
	Phoenix Integrated Surgical Residency Faculty lectures:
	Acute Arterial Occlusion, 1991
	Safe Carotid Endarterectomy, 1993
	Surgical Considerations in Diabetic Ischemia, 1994
	Texas Surgical Society:
	Early Experiences in Distal Bypass Surgery for Patients with

EXHIBIT
B

DEFINITIONS
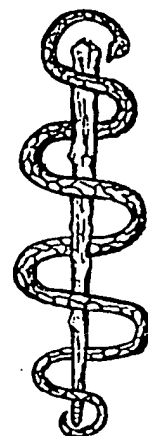
STEDMAN'S MEDICAL DICTIONARY

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*A vocabulary of medicine and
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and derivations*

TWENTY-SECOND EDITION

*Completely revised by a staff of 33 editors, covering
44 specialties and subspecialties*

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anxiety accompanying psychosomatic disorders; should not be used for nausea of pregnancy.

bud. A structure that resembles the b. of a plant.

bronchial b., one of the outgrowths from the primordial bronchus responsible for the continued ramification of the embryonic bronchial tree.

end b., tail b.

farcy b., one of a number of nodules formed along the course of the subcutaneous lymphatics in cases of glanders.

gustatory b., *caliculus* gustatorius.

liver b., the primordial cellular outgrowth from foregut entoderm of the embryo that gives rise to the parenchyma of the liver.

lung b., in the embryo, one of the two lateral outgrowths from the respiratory primordium that ultimately forms the epithelial portions of the lung.

metanephric b., ureteric b.; the primordial cellular outgrowth from the mesonephric duct that gives rise to the epithelial lining of the ureter, pelvis and calyces of the kidney, and the straight collecting tubules.

syncytial b., syncytial *knot.*

tail b., end b.; the rapidly proliferating mass of cells at the caudal extremity of the embryo.

taste b., *caliculus* gustatorius.

tooth b., the primordial structures from which a tooth is formed; the enamel organ, dental papilla, and the dental sac enclosing them.

ureteric b., metanephric b.

vascular b., an endothelial sprout arising from a blood vessel.

Budd, George, London physician, 1808-1882. See B.'s *cirrhosis, jaundice, syndrome.*

Budde (bood'deh), E., Danish sanitary engineer, *1871. See B. *process.*

buddeize (bood'de-ize). To treat by the Budde process.

budding. Gemmation.

Budge (bood'ga), Julius L., German physiologist, 1811-1888. See B.'s *center.*

Budin (bü-dän'), Pierre C., French gynecologist, 1846-1907. See B.'s obstetrical *joint,* B.'s *pelvimeter.*

Buerger, Leo, New York physician, 1879-1943. See B.'s *disease,* Winiwarter-B. *disease,* B.'s *stain.*

bufa-, bufo-. Combining forms that denote origin from toads. They are used in the systematic and trivial names of a great number of toxic substances (genins) isolated from plants and animals containing the bufanolide structure (see bufanolide). Prefixes denoting species origin are often attached, *e.g.,* marinobufagin, marinobufotoxin.

bufagenins. Bufagins.

bufagins. Bufagenins; a group of steroids (bufanolides) in the venom of a family of toads, the Bufonidae, having a digitalis-like action upon the heart (*e.g.,* bufotalin); *cf.* bufotoxins. For structure of bufanolides, see steroids.

bufalin. A specific type of bufanolide, containing 3β,14-dihydroxy-5β,14β-bufa-20,22-dienolide. For structure of bufanolide, see steroids.

bufanolide. Fundamental steroid lactone of several squill-toad (Bufonidae) venoms or toxins; also found in the form of glycosides in plants (*cf.* digitalis). The steroid is essentially that of 5β-androstane, with a 14β-H. The lactone at C-17 is structurally related to —CH(CH₃)CH₂CH₂CH₃ radical attached to C-17 in the cholanes, and is in the same configuration as that of cholesterol (*i.e.,* 20R); in some species, b. is formed from cholesterol. Various b. derivatives having unsaturation in the lactone ring (20,22) or elsewhere (4) are known as bufenolides (one double bond), bufadienolides (*e.g.,* bufalin, telecinobufagin, marinobufagin, bufogenin B, bufotalin, bufotoxin), bufatrienolides (*e.g.,* scillarenin), etc. They have varying numbers of hydroxyl groups at positions 3, 5, 14, and 16, and these may be further substituted (*e.g.,* bufatalin, bufotoxin, gitoxigenin). For structure, see steroids.

buffer. 1. A mixture of an acid and its conjugate base (salt), such as H₂CO₃/HCO₃⁻; H₂PO₄⁻/H₂PO₄⁻, which when present in a solution reduces any changes in pH that would otherwise occur in the solution when acid or alkali is added to it. Thus the pH of the blood and body fluids is maintained virtually constant (pH 7.45)

although acid metabolites are continually being formed in the tissues and CO₂(H₂CO₃) is lost in the lungs. See also conjugate acid-base pair, under conjugate. 2. To add a b. to a solution and thus give it the property of resisting a change in pH when it receives a limited amount of acid or alkali.

b. capacity, the amount of hydrogen ion (or hydroxyl ion) required to bring about a specific pH change in a specified volume of a b. (see b. value).

b. pair, an acid and its conjugate base (anion).

secondary b., see Hamburger's *law.*

b. value, the power of a substance in solution to absorb acid or alkali without change in pH; this is highest at a pH equal to the pK of the acid of the b. pair (see b. capacity).

b. value of the blood, the ability of the blood to compensate for acid-alkali fluctuations without disturbance of the pH.

Buffon (boo'-fon), Compt de (Georges Louis Leclerc), French naturalist, 1707-1788. Published *Histoire Naturelle.* Some of his views on evolution and the origin of species anticipated Darwin by more than a hundred years.

buffy coat. Crusta inflammatoria; crusta phlogistica; the upper, lighter portion of the blood clot (coagulated plasma and white blood cells), occurring when coagulation is delayed so that the red blood cells have had time to settle a little; the portion of centrifuged, anticoagulated blood which contains leukocytes and platelets.

bufo-. See bufa-.

bufogenin B. A steroid toxin from Chinese toads; a 3β,14,16-trihydroxy-bufa-20,22-dienolide; *cf.* bufalin.

Bufonidae [L. *bufo,* toad]. A family of toads whose dermal glands secrete several kinds of pharmacologically active substances having a cardiac action similar to that of digitalis.

bufotalin. The steroid of a bufotoxin (bufogenin). It is bufogenin B acetylated at the C-16 OH.

bufoten'ine. Mappine; 3-(2-dimethylaminoethyl)indol-5-ol; N,N-dimethylserotin; a psychotomimetic agent isolated from the venom of certain toads. It raises the blood pressure by a vasoconstrictor action and produces psychic effects including hallucinations. It is also present in several plants and is one of the active principles of cohoba.

bufotox'in. Vulgarobufotoxin; a toxic substance in venom of *Bufa vulgaris,* the common European toad; bufotalin esterified with suberylarginine at C-14 OH group.

bufotox'ins. A group of steroid lactones (conjugates of bufogenins and suberylarginine at C-14) of digitalis present in the venoms of the Bufonidae. Their effects are similar to but weaker than those of the bufagins.

bug'gery [O.F. *bougre,* heretic]. Bestiality; sodomy.

Buhl (bool), Ludwig von, German pathologist, 1816-1880. See B.'s *disease.*

Buist, Robert C., Scottish obstetrician, 1860-1939. See B.'s *method.*

bulb [L. *bulbus,* a bulbous root]. 1. Any globular or fusiform structure. 2. *Medulla* oblongata. 3. A short, vertical underground stem of plants such as scilla and allium.

aortic b., *bulbus* aortae.

arterial b., *bulbus* aortae.

carotid b., *sinus* caroticus.

b. of corpus spongiosum, *bulbus* penis.

dental b., the papilla, derived from mesoderm, that forms the part of the primordium of a tooth which is situated within the cup-shaped enamel organ.

duode'nal b., duodenal *cap.*

end b., one of the oval or rounded bodies in which the sensory nerve fibers terminate in mucous membrane.

b. of eye, *bulbus* oculi.

hair b., *bulbus* pili.

ju'gular b., *bulbus* venae jugularis.

Krause's end b., *corpusculum* bulboideum.

b. of lateral ventricle, a rounded elevation in the dorsal part of the medial wall of the posterior horn of the lateral ventricle, produced by the forceps major.

olfac'tory b., *bulbus* olfactorius.

b. of penis, *bulbus* penis.

rachid'ian b., *medulla* oblongata

Rouget's b., a venous pl

taste b., *caliculus* gustatc

b. of ure'thra, *bulbus* pe

b. of vestibule, *bulbus* v

bulbar. 1. Relating to a bull medulla oblongata.

bulbi'tis. Inflammation of

bulbocap'nine [G. *bolbos,* An alkaloid from *Corydalis Fumariaceae*). Produces a s recommended in the treatmen disease, paralysis agitans, an

bul'bocaverno'sus. See u

bulboid [G. *bolboeidēs,* fr resemblance]. Bulb-shaped.

bulbonu'clear. Relating to

bulbopon'tine. Denoting t pons and the region of the m

bulbosac'ral. Relating to segments of the spinal cord.

bulbospin'al. Relating to th particularly to nerve fibers in

bulbourethral (bul'bo-u-re'

bulbus, gen. and pl. bull

b. aor'tae [NA], aortic bult dilation where the truncus arte

b. cordis, b. aortae.

b. cornus posterior'is [N, lateral ventricle of the brain; ε of the posterior horn produce(of the corpus callosum as the lobe.

b. oc'uli [NA], bulb of the c proper without the appendage

b. olfacto'rius [NA], olfa anterior extremity of the olfac plate of the ethmoid and rece

Olfactory bulb

Cribriform plate of ethmoid

Olfactory mucosa

Bulbus O

Diagram of olfactory muc y Cajal), showing neuronal rel Copenhaver, W. F.: *Bailey's* The Williams & Wilkins Co.,

b. penis [NA], bulb of corpus **b. urethrae;** the expanded poster penis lying in the interval betwe

b. pili [NA], hair bulb; the lo that fits like a cap over the pap follicle.

b. ure'thrae, b. penis.

b. venae jugula'ris [NA], b dilated parts of the internal jugu at the beginning of the internal j

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Thesaurus

    bud[1,noun]
    bud[2,verb]
Go To   bud scale
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Main Entry: ¹**bud**
Pronunciation: 'b&d
Function: *noun*
Etymology: Middle English *budde*
Date: 14th century
1 : a small lateral or terminal protuberance on the stem of a plant that may develop into a flower, leaf, or shoot
2 : something not yet mature or at full development: as **a** : an incompletely opened flower **b** : CHILD, YOUTH **c** : an outgrowth of an organism that differentiates into a new individual : GEMMA; *also* : PRIMORDIUM
- **in the bud** : in an early stage of development in the bud>

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* generally or often considered vulgar
|| usage restricted; consult a dictionary for more information

For further explanation of these symbols see the Thesaurus Symbol Guide.

Dictionary Pronunciation Key

(Merriam-Webster)

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> Thesaurus

Main Entry: **pri·mor·di·um**
Pronunciation: ~dE-&m
Function: *noun*
Inflected Form(s): *plural* **pri·mor·dia** /-dE-&/
Etymology: New Latin, from Latin
Date: 1671
: the rudiment or commencement of a part or organ

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For further explanation of these symbols see the Thesaurus Symbol Guide.

Dictionary Pronunciation Key

- \&\ as a and u in abut
- \&\ as e in kitten
- \&r\ as ur and er in further
- \a\ as a in ash
- \A\ as a in ace
- \ä\ as o in mop
- \au\ as ou in out
- \ch\ as ch in chin
- \e\ as e in bet
- \E\ as ea in easy
- \g\ as g in go
- \i\ as i in hit
- \I\ as i in ice
- \j\ as j in job
- \[ng]\ as ng in sing
- \O\ as o in go
- \o\ as aw in law
- \oi\ as oy in boy
- \th\ as th in thin
- \th\ as th in the
- \ü\ as oo in loot
- \u\ as oo in foot
- \y\ as y in yet
- \zh\ as si in vision

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organogenesis

organogenesis,

in embryology, the series of organized integrated processes that transforms an amorphous mass of cells into a complete organ in the developing embryo. The cells of an organ-forming region undergo differential development and movement to form an organ primordium, or anlage. Organogenesis continues until the definitive characteristics of the organ are achieved. Concurrent with this process is histogenesis; the result of both processes is a structurally and functionally complete organ. The accomplishment of organogenesis ends the period during which the developing organism is called an embryo and begins the period in which the organism is called a fetus. See also histogenesis.

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  organ                               ▲
  barrel organ
  electric organ                      ▼
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Main Entry: **organ**
Pronunciation: 'or-g&n
Function: *noun*
Etymology: Middle English, partly from Old English *organa,* from Latin *organum,* from Greek *organon,* literally, tool, instrument; partly from Old French *organe,* from Latin *organum;* akin to Greek *ergon* work -- more at WORK
Date: before 12th century
1 a *archaic* : any of various musical instruments; *especially* : WIND INSTRUMENT **b** (1) : a wind instrument consisting of sets of pipes made to sound by compressed air and controlled by keyboards and producing a variety of musical effects -- called also *pipe organ* (2) : REED ORGAN (3) : an instrument in which the sound and resources of the pipe organ are approximated by means of electronic devices (4) : any of various similar cruder instruments
2 a : a differentiated structure (as a heart, kidney, leaf, or stem) consisting of cells and tissues and performing some specific function in an organism **b** : bodily parts performing a function or cooperating in an activity organs>
3 : a subordinate group or organization that performs specialized functions organs of government>
4 : PERIODICAL

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Thesaurus Symbol Key

* generally or often considered vulgar
|| usage restricted; consult a dictionary for more information

EXHIBIT C

EXHIBIT C
SUMMARY OF MATERIALS

EXH. NO.	MATERIAL AND DATE	SOFT TISSUE	TECHNIQUE	GROWTH FACTOR
C-1	Science Daily (American Heart Association), 1998, "Study is first ever to document protein therapy induces creation of new blood vessels to the human heart" SYNOPSIS: For the first time ever, growth factor inserted into the body grows a new vascular system.	Blood vessels to heart	Injection	Human recombinant basic fiberblast growth factor (genetically manipulated and produced)
C-2	Circulation, 1998, "Induction of neoangiogenesis in ischaemic myocardium by human growth factors: first clinical results of a new treatment of coronary heart disease" SYNOPSIS: A new therapeutic concept and followup tests confirm a true de novo vascular system was formed . Vascular buds consisting of endothelial sprouts (capillaries) were created. The capillaries grew further and differentiated into two-layered metarterioles. The process of organogenesis continued with the metarterioles differentiating into three-layered arterioles (arteries).	Blood vessels to heart	Injection	Human recombinant basic fiberblast growth factor (genetically manipulated and produced)

EXH. NO.	MATERIAL AND DATE	SOFT TISSUE	TECHNIQUE	GROWTH FACTOR
C-3	<u>Circulation</u>, 1998, Editorial, "Angiogenic therapy of the human heart" <u>SYNOPSIS</u>: Basic research in a different field (cancer) purified angiogenic growth factors in the 1980's. A novel clinical application of these growth factors introduces a new modality-the regulation of blood vessel growth.	Editorial	Editorial	Editorial
C-4	<u>NIH Press Release</u>: 1999, "Growing New blood vessels with a timed-release capsule of growth factor is a promising treatment for heart bypass patients, finds NHLBI Study" <u>SYNOPSIS</u>: Researchers at Harvard Mecial School inserted timed-release capsules of basic fibroblast growth factor into [human] heart muscle to grow new blood vessels.	Blood vessels to heart	Insertion of timed-release capsule	Basic fibroblast growth factor
C-5	<u>The Lancet</u>, 1996, "Clinical Evidence of angiogenesis after arterial gene transfer of phVEGF in Patient with Ischaemic limb" <u>SYNOPSIS</u>: Growth factor plus living material (plasmid) inserted into the body with a gel carrier to grow new blood vessels in the leg of a patient.	Blood vessels to leg	Balloon Catheter/ hydrogel	Vascular endothelial growth factor plus living material (plasmid)

EXH. NO.	MATERIAL AND DATE	SOFT TISSUE	TECHNIQUE	GROWTH FACTOR
C-6	U.S. Patent No. 5,652,225 (1997) Parent application filed 10/04/94 SYNOPSIS: The formation of new blood vessels in a human host by inserting a growth factor with a carrier into the body.	Formation of new blood vessels	Balloon catheter/ hydrogel	Angiogenic growth factors
C-7	Harvard University Gazette, 1998, "New Arteries Grown in Diseased Hearts" SYNOPSIS: Harvard Medical School researchers inject basic fibroblast growth factor through a carrier (tube) to grow new arteries in a human heart.	Formation of new arteries in hearts	Injection via tube (catheter); and implanted timed-release capsules	Basic fibroblast growth factor

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Study Is First Ever To Document Protein Therapy Induces Creation Of New Blood Vessels To The Human Heart

DALLAS, Feb. 24 -- For the first time, scientists have published research evidence that recombinant protein therapy can create new blood vessels to increase blood supply to the human heart. The report from German scientists appears in today's Circulation: Journal of the American Heart Association.

FGF-I, a human growth factor obtained through genetic engineering, was used in 20 patients with some form of ischemic or coronary heart disease, which results from blockages in the vessels leading to and from the heart. By injecting the growth factor near the blocked vessels, the scientists were able to induce neoangiogenesis -- the process by which the body can grow its own new capillary network to bypass occluded vessels.

"This capillary network is a true de novo vascular system," says Thomas-Joseph Stegmann, M.D., head of the department of thoracic and cardiovascular surgery at the Fulda Medical Center, Fulda, Germany. "We were able to use the recognized physiological effects of FGF-I to induce neoangiogenesis in the human ischemic heart."

As early as four days after application of FGF-I, the vascular structure around the diseased vessels was completely altered in all 20 of the patients. Like the spokes of a bicycle wheel, the new capillary vessels radiated outward from the point of injection, resulting in a twofold to threefold increase in blood flow to the heart, says the study's lead author.

Researchers found, on average, the ejection fraction of the 20 patients improved from 50.3 percent to 63.8 percent in the three years following the procedure. Ejection fraction measures how much blood leaves the

heart with each beat and indicates how well the left ventricle -- the heart's main pumping chamber -- is functioning.

In follow-up angiographic imaging of the patients, it was clear that the growth factor injection had stimulated the creation of a new vascular system, says Stegmann. Three months after the procedure, he and his colleagues examined angiograms -- X-ray images of the heart -- of both the treated and control (untreated) patients and found that no blockages had formed in the new vessels.

All of the patients who received the FGF-I three years ago are still alive. The scientists report that no negative side effects have been seen in the patients who received the FGF-I.

Elizabeth Nabel, M.D., an American Heart Association board member, has done extensive research in gene and recombinant protein therapy over the past 12 years. She says this new research is encouraging for cardiovascular surgeons.

"It's a very important therapy for patients who have blocked arteries that are not amenable to bypass," says Nabel, professor of internal medicine and physiology and chief, division of cardiology at the University of Michigan. "This is not to say that bypass should be abandoned, but this research shows angiogenesis is a powerful therapy to be used with bypass surgery."

The procedure is still experimental, but scientists say the use of FGF-I may particularly benefit patients whose blocked vessels cannot be treated by cardiac bypass operations.

"At the moment, this procedure could not replace conventional bypass surgery," says Stegmann. "The question remains to be answered whether FGF-I or other growth factors are able to treat occlusions of greater coronary vessels, but currently, this is not possible."

Scientists have used gene therapy to grow vessels in other parts of the body -- such as in the legs in order to improve the health of patients who have blockages in lower leg blood vessels -- but this is the first published account of the use of recombinant protein therapy to induce angiogenesis in human hearts.

FGF-I was obtained from strains of Escherichia coli by genetic engineering, then isolated and highly purified the recombinant FGF-I protein. After several series of animal experiments demonstrated the potency of FGF-I, it was used in humans for the first time.

When scientists create recombinant protein, they take the DNA of a growth factor (in this case FGF-I) and manipulate it into RNA (ribonucleic acid) by growing it in bacteria cultures in the laboratory. RNA is then manufactured into protein, which is isolated and purified

before it is injected into the hearts of patients.

Twenty patients -- 14 men and 6 women who were at least 50 years old --
who had no prior history of heart attack or cardiac surgery had an
operation to clear blockages in more than one vessel. All of them had
stenosis -- narrowed blood flow due to atherosclerosis -- in their internal
mammary artery/left anterior descending coronary artery. During the
operative procedure, the growth factor protein -- in a dosage of 0.01
milligrams per kilogram of body weight -- was directly injected into the
heart muscle near the blockage.

Prior to using the treatment in humans, the scientists performed several
series of animal experiments, most specifically in ischemic rat hearts.
Having found that the FGF-I injection worked in those animal models, the
researchers theorized that it would also work in humans.

Study co-authors are P. Pecher, M.D.; B.U. von Specht, M.D. and B.
Schumacher, M.D.

Clinical Investigation and Reports

Induction of Neoangiogenesis in Ischemic Myocardium by Human Growth Factors

First Clinical Results of a New Treatment of Coronary Heart Disease

B. Schumacher, MD; P. Pecher, MD; B.U. von Specht, MD; Th. Stegmann, MD

Background—The present article is a report of our animal experiments and also of the first clinical results of a new treatment for coronary heart disease using the human growth factor FGF-I (basic fibroblast growth factor) to induce neoangiogenesis in the ischemic myocardium.

Methods and Results—FGF-I was obtained from strains of *Escherichia coli* by genetic engineering, then isolated and highly purified. Several series of animal experiments demonstrated the apathogenic action and neoangiogenic potency of this factor. After successful conclusion of the animal experiments, it was used clinically for the first time. FGF-I (0.01 mg/kg body weight) was injected close to the vessels after the completion of internal mammary artery (IMA)/left anterior descending coronary artery (LAD) anastomosis in 20 patients with three-vessel coronary disease. All the patients had additional peripheral stenoses of the LAD or one of its diagonal branches. Twelve weeks later, the IMA bypasses were selectively imaged by intra-arterial digital subtraction angiography and quantitatively evaluated. In all the animal experiments, the development of new vessels in the ischemic myocardium could be demonstrated angiographically. The formation of capillaries could also be demonstrated in humans and was found in all cases around the site of injection. A capillary network sprouting from the proximal part of the coronary artery could be shown to have bypassed the stenoses and rejoined the distal parts of the vessel.

Conclusions—We believe that the use of FGF-I for myocardial revascularization is in principle a new concept and that it may be particularly suitable for patients with additional peripheral stenoses that cannot be revascularized surgically. (*Circulation*. 1998;97:645–650.)

Key Words: growth substances ■ angiogenesis ■ coronary disease

For the cardiac surgeon who is attempting to treat CHD, the use of sections of autologous blood vessels as bypass material is subject to severe limitations. Autologous arterial conduits are in short supply, and segments of the saphenous vein do not remain patent for very long.[1,2] Furthermore, "complete" revascularization is limited if diffuse coronary arteriosclerosis is present and extensive, especially if there are additional peripheral stenoses.

In the search for alternative and/or additional treatment for improving the long-term prognosis, especially in diffuse CHD, attention has recently been directed toward natural angiogenesis.[3,9] Growth factors, especially FGF-I, have recently become of major importance because they can induce angiogenesis.[8,10–12]

Gimenez-Gallego et al[13] succeeded in elucidating the biochemical structure of FGF-I in 1985. Jaye et al[14] isolated human FGF-I from brain tissue in 1986. In 1991, Forough and coworkers[15] successfully used the technique of gene transfer to introduce the information for expressing human FGF-I into apathogenic *Escherichia coli*.

Our aim was to evaluate the information currently available on the biological effect of angiogenetic growth factors in animals and, if appropriate, to use human growth factor for the treatment of CHD. This involved (1) the production of human growth factor by genetic engineering, followed by its isolation, characterization, and purification; (2) using animal experiments to establish its angiogenetic potency and to exclude any possible pathogenic effect; and (3) using FGF-I clinically as an adjunct to coronary surgery and to demonstrate neoangiogenesis in the ischemic human myocardium.

Methods

Production and Purification of FGF-I

The production and purification of human FGF-I is a biochemically elaborate technique. The individual experimental steps have been reported elsewhere.[4,7]

Genetic engineering was used to produce human FGF-I from apathogenic strains of *E coli*, a plasmid containing the genetic information being introduced into the microorganisms.[15] These were kindly provided by Prof T. Maciag (Laboratory of Molecular Biology, American Red Cross, Rockville, Md). After production, FGF-I was eluted by heparin sepharose column chromatography, and several elution fractions were collected and purified by dialysis. Positive protein elution fractions were identified in the BIO-RAD assay[7] by SDS-PAGE,[16] and the biochemical isolation of FGF-I was confirmed by the Western blot method.[17] Further purification was obtained by HPLC.[18] The factors were lyophilized and stored at −32°C and diluted to 1 mL with NaCl solution containing 500 IU of heparin.

EXHIBIT C-2

Chorioallantoic Membrane Assay

This established method, which provides a direct demonstration of the effect of growth factors on living tissue, was used to investigate the angiogenetic effect of FGF-I.[19,20] The growth of the allantoic systems can be directly observed by light microscopy. After incubation of 20 fertilized hen eggs for 13 days, the growth factor was applied to the membrane and covered with tissue culture coverslips. Four days later, the membrane was examined under the light microscope and directly compared with controls untreated with FGF-I or treated with heat-denatured FGF-I (70°C for 3 minutes).

Exclusion of the Pyrogenicity of FGF-I

Varying concentrations of FGF-I (0.01, 0.5, or 1.0 mg/kg body weight) were injected subcutaneously, intramuscularly, or intravenously into 27 New Zealand White rabbits, the solvent alone being used for an additional 13 controls. Thereafter, the rectal temperature was taken every half hour for 3 hours, hourly for the rest of the day, and every 8 hours for 12 days. A daily white cell count was also repeated for 12 days (see "Results"). In addition to this, the erythrocyte sedimentation rate and the C-reactive protein values were determined on the 3rd, 6th, 9th, and 12th days after the injection.

Confirmation of the Angiogenetic Potency of FGF-I in Animal Experiments

Supplementary to our earlier experiments,[4,7] the effect of FGF-I was also investigated in the ischemic hearts of inbred Lewis rats (a total of 275 animals, including 125 controls treated with heat-denatured FGF-I, 70°C for 3 minutes). The pericardium was opened via the abdominal wall and diaphragm, and two titanium clips were inserted at the apex of the left ventricle to induce myocardial ischemia. Growth factor (mean concentration of 10 μg) was then injected locally into the site. The coronary vessel system was imaged by aortic root angiography after 12 weeks and, finally, a specimen from the same myocardial region was evaluated histologically.

Clinical Use of FGF-I in Patients With CHD

This study was approved by the Medical Research Commission at the Phillips University of Marburg on August 10, 1993 (No. 47/93). This is the usual ethics commission for our hospital. Twenty patients without any history of infarction or cardiac surgery (14 men and 6 women; minimum age, 50 years) were subjected to an elective bypass operation for multivessel coronary heart disease. The growth factor was applied directly during the operation. As a control group, 20 patients who underwent the same procedure were given heat-denatured FGF-I (70°C for 3 minutes). The choice of treatment was completely random, the names being placed in sealed envelopes and selected in a blinded manner.

The details, nature, and aims of this procedure were explained beforehand to every patient who underwent the operation. In all cases, we received their fully informed consent. Both groups of patients were closely comparable with regard to clinical symptoms, accompanying disorders, cardiovascular risk factors, ventricular function, sex, and age. A comparable coronary morphology was found in both groups.

All patients had a further stenosis in the distal third of the LAD or at the origin of one of its branches in addition to a severe proximal stenosis. The mean ejection fraction of the left ventricle for all patients was 50%. The operative procedure for coronary revascularization with autologous grafts (an average per patient of 2 to 3 venous bypasses and 1 from the left IMA) was routinely performed. FGF-I (mean concen-

Figure 1. Intraoperative administration of growth factor.

tration, 0.01 mg/kg body weight) was injected into the myocardium, distal to the IMA/LAD anastomosis and close to the LAD, during the maintenance of the extracorporeal circulation and after completion of the distal anastomoses (Fig 1). In the control group, heat-denatured FGF-I was substituted for FGF-I. After 12 weeks, the IMA bypasses of all the patients were imaged selectively by transfemoral, intra-arterial, and digital subtraction angiography.

Angiograms obtained in this way were evaluated by means of EDP-assisted digital gray-value analysis, a universally recognized and well-established technique for demonstrating capillary neoangiogenesis.[21-26] Sites of interest both with and without FGF-I (meaning heat-denatured FGF-I) were selected in the vessels filled with contrast medium and in regions of the myocardium distal to the IMA/LAD anastomosis. One hundred pixels were selected from each site of interest and analyzed digitally. Complete blackening of the x-ray films was rated with a gray value of 150, and areas without blackening of the film were allotted a zero value. During the first 5 postoperative days, separate laboratory checks in addition to the routine postoperative follow-up procedures were made twice daily, and the temperature checked three times a day.

Results

After separation, purification, and stabilization, we were able to isolate human FGF-I in all 40 bacterial cultures and demonstrate its high degree of purity. Fig 2 shows an HPLC profile of the growth factor after routine purification. The peak values at the beginning and end of the profile represent impurities that could be identified as E coli proteins. FGF-I could be further separated by fractionated collection, and the control HPLC (Fig 3) merely shows the peak value of this fraction on an otherwise even baseline.

Figure 2. HPLC profile before high purification. HBGF-I indicates human FGF-I; %E, extinction.

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Figure 3. HPLC profile after high purification. HBGF-I indicates human FGF-I; %E, extinction.

In the chorioallantoic membrane assay, the angiogenetic potency of FGF-I could be demonstrated in vivo. As early as 4 days after application of the factor, the vascular structure of the membrane was completely altered. Emanating radially from the site of application, an unequivocal growth of new vessels from the original host vessels had grown out into the periphery (Fig 4A). These structures were completely absent from the control group, and a normally developed reticular vascular pattern could be discerned (Fig 4B).

Pyrogenic effects of the human growth factor produced in this way could be definitively ruled out in the animal model. There was no significant rise of body temperature when checked at short intervals and no trace of an inflammatory reaction in comparison with the control group (n=13) in any of the 27 test animals during the period of observation. This result was independent of the concentration and the route of administration (intravenous, subcutaneous, or intramuscular) of the factor.

Earlier investigations into the application of FGF-I to the nonischemic rat heart made it possible to demonstrate neoangiogenesis both histologically and angiographically after 9 weeks in 11 of 12 test animals after the implantation of a tissue bridge pretreated with growth factor between the heart and thoracic aorta. In the control group without FGF-I (n=6), no signs of induced neoangiogenesis could be found.[4,7]

Unequivocal proof of induced neoangiogenesis was also found in the ischemic rat heart. In the test animals, in which myocardial ischemia had previously been induced with titanium clips and growth factor had subsequently been injected into the myocardium, a manifest accumulation of contrast medium was shown by aortic angiography at the site of the FGF-I injection 12 weeks later (Fig 5A), whereas such an accumulation of contrast medium did not appear in any of the control animals (Fig 5B). Histological examination of the myocardium revealed a threefold increase in the capillary density per square millimeter around the site of the FGF-I injection.

When the growth factor FGF-I was used clinically for the first time on the human heart, neoangiogenesis together with the development of a normal vascular appearance could be demonstrated angiographically, exactly as in the earlier animal experiments.[4,7] Selective imaging of the IMA bypasses by intra-arterial digital subtraction angiography confirmed the following result in all 20 patients: at the site of injection and in the distal areas supplied by the LAD, a pronounced accumulation of contrast

10 ng HBGF-I

without HBGF-I

Figure 4. A, Chorioallantoic membrane assay with application of the growth factor. B, Chorioallantoic membrane assay of the control group. HBGF-I indicates human FGF-I.

medium extended peripherally around the artery for ~3 to 4 cm, distal to the IMA/LAD anastomosis (Fig 6A). In the control angiograms of patients to whom only heat-denatured FGF-I had been given, the IMA/LAD anastomosis was also recognizable, but the accumulation of contrast medium described above was absent (Fig 6B). The angiograms of both the treated and control groups were recorded at a rate of four images per second, and these show

10 µgHBGF-I

10 µg/kg HBGF-I

without HBGF I

Figure 5. A, Administration of the growth factor in ischemic rat heart with a clearly discernible accumulation of contrast medium at the site of injection. B, No discernible accumulation of contrast medium in the control group. HBGF-I indicates human FGF-I.

comparable distances between the beginning of the injection and visualization of the medium.

At the site of injection of the FGF-I, a capillary network could be seen sprouting out from the coronary artery into the myocardium. This enabled retrograde imaging of a stenosed diagonal branch to be performed (Fig 7A). Such "neocapillary vessels" can also provide a collateral circulation around additional distal stenoses of the LAD (Fig 7B) and bring about

without HBGF-I

Figure 6. A, Angiography after injection of the growth factor into the human heart shows a pronounced accumulation of contrast medium compared with the control group. B, Angiography in the control group does not show any increased accumulation of contrast medium around the IMA/LAD anastomosis. HBGF-I indicates human FGF-I.

retrograde filling of a short segment of the artery distal to the stenosis. In none of the angiograms of the treated patients taken 12 weeks after the operation were any new stenoses of the LAD detectable.

The results of EDP-assisted digital gray value analysis for quantification of the neoangiogenesis (Fig 8) gave a mean gray value of 124 for the vessels. The control myocardium reached

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Figure 7. A, Collateralization of stenoses (arrow): a diagonal branch occluded just distal to its origin is filled through the newly grown capillaries. B, Collateralization of stenoses (arrow) by newly grown capillaries: the peripherally stenosed LAD is filled through these vessels. HBGF-I indicates human FGF-I.

a gray value of only 20, and that of the myocardium injected with FGF-I gave a value of 59 (Fig 8).

Discussion

Normal capillaries have a cell population with a low turnover rate of months or years. On occasion, however, a high turnover rate of this cell population is possible even under physiological

Figure 8. Quantitative gray value analysis of contrast medium accumulation in the angiography shows a twofold to threefold increase in the local blood flow at the site of injection. HBGF-I indicates human FGF-I.

conditions, and this naturally leads to the rapid growth of new capillaries and other blood vessels. Such a physiological process occurs in the development of the placenta, in fetal growth, and in wound healing, as well in the formation of collaterals in response to tissue ischemia. "Angiogenetic growth factors," which are biochemically polypeptides, are essential for such processes as capillary growth or neoangiogenesis. These growth factors (for instance, the human heparin-binding FGF-I) bring about their effect by significantly increasing cell proliferation, differentiation, and migration via a high-affinity receptor system on the surfaces of the endothelial cells.[8,10-12]

During the last few years, several working groups have been able to establish indications for the effective use of growth factors to improve blood flow in the presence of tissue ischemia in animal experiments.[3,9,27] Yanagisawa-Miwa et al[9] succeeded in demonstrating a significant collateralization together with reduction in the size of the infarct after intracoronary administration of growth factor in rabbits. Baffour et al[3] also observed a significant formation of collaterals in ischemic extremities after growth factor administration in animals. Albes et al[27] produced a distinct improvement in the blood flow in ischemic tracheal segments implanted subcutaneously in rabbits by injecting growth factor–enriched fibrin glue locally.

After growth factor was injected into the ischemic rat heart,[4,7] we were able to observe induced neoangiogenesis and confirm it angiographically. We were also able to prove histologically that this neoangiogenesis brings about the development of new vascular structures with a three-layered vessel wall. Angiographic imaging confirmed that these are anatomically normal capillaries and other blood vessels.

The production of human FGF-I by our molecular biological method has proved to be a complex but readily reproducible procedure. From the bacterial cultures, we are able to isolate the factor as a pure substance in sufficient quantities. By in vitro assay and as a result of extensive animal experiments, we were able to exclude the possible pyrogenic effects of FGF-I.

In earlier animal experiments,[4] we were able to demonstrate the proliferative and mitogenic effects of the growth factor on human saphenous vein endothelial cells. Endothelial cell cultures with added growth factor induced a confluent monolayer after only 5 to 9 days, whereas the monolayer was not complete before 7 to 11 days in the control group. In addition to determining the total cell count with a cell counter, we also confirmed this result by analyzing the rate of DNA synthesis by measuring the incorporation of ³H-thymidine into the endothelial cell nuclei using the

method of Klagsbrun and Shing.[28] The cell proliferative potency of FGF-I could be further intensified by adding heparin, a glycosaminoglycan protecting the growth factor from inactivation by cellular enzymes and from heat and chemical denaturation.[29]

On the basis of these in vitro and in vivo experiments, we established for the first time the efficacy of FGF-I for the treatment of CHD, and were able to demonstrate that it can induce neoangiogenesis in situ in the ischemic human heart. This possibility has been widely discussed for many years but never before attempted.

A dense capillary network appeared around the site of injection of the factor in the myocardium of all our treated patients. This capillary network is a true de novo vascular system. Emerging from the proximal segment of the LAD, it sprouts out into the surrounding myocardium, bringing about a twofold to threefold increase in the local blood supply through these newly formed functional vessels. We were able to use the recognized physiological effects of FGF-I (as they occur in the repair mechanism of wound healing or in collateralization of ischemic tissue) to induce neoangiogenesis in the human ischemic heart.

We also consider that administration of FGF-I (produced in this way by genetic engineering), combined with operative myocardial revascularization, may well be an especially appropriate treatment for patients with additional peripheral stenoses that cannot be treated surgically.

In our opinion, neoangiogenesis induced by FGF-I opens up new possibilities for the treatment of ischemic myocardial disease. Furthermore, it could become a new therapeutic concept in the management of diffuse CHD after alternative methods of administration have also been developed. This method of inducing neoangiogenesis is also conceivable as a therapeutic option in other regions of the cardiovascular system in which arterial occlusion has led to ischemia.[30] However, before any such possibilities are realized, many more clinical investigations will have to be performed.

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Angiogenic Therapy of the Human Heart

Judah Folkman, MD

The field of angiogenesis research was initiated 27 years ago by a hypothesis that tumors are angiogenesis-dependent.[1] Shortly thereafter, in the early 1970s, it became possible to passage vascular endothelial cells in vitro for the first time.[2] Bioassays for angiogenesis were developed subsequently throughout that decade. The early 1980s saw the purification of the first angiogenic factors.[3-6] By the mid-1980s, angiogenesis inhibitors began to be discovered.[7-9] Translation of these laboratory findings to clinical application started in 1989, when interferon alfa was first used for the treatment of life-threatening hemangiomas in infants.[10-12]

Clinical applications of angiogenesis research are being pursued along three general lines: (1) prognostic markers in cancer patients,[13,14] (2) antiangiogenic therapy (for review, see Reference 15), and (3) angiogenic therapy. The first angiogenic therapy of ischemic vascular disease was the administration of vascular endothelial growth factor (VEGF)/vascular permeability factor to patients with severe peripheral vascular disease in the lower limbs.[16]

In a landmark paper, Schumacher and colleagues now report the first angiogenic therapy of human coronary heart disease.[17] It is an important study, not only because the authors describe how they produced their own recombinant human fibroblast growth factor-1 (FGF-1, also called acidic fibroblast growth factor) and tested it in vitro and in vivo but also because they conducted a randomized controlled clinical trial. In 20 patients with three-vessel coronary artery disease who underwent two or three venous bypass grafts and one from the internal mammary artery, the angiogenic protein FGF-1 was injected into the myocardium close to the left anterior descending coronary artery and distal to its anastomosis with the internal mammary artery. FGF-1 was injected during extracorporeal surgery and again after completion of the anastomosis. Trans-femoral, intra-arterial digital subtraction angiography 12 weeks later showed coronary artery neovascularization extending out from the area of FGF-1 injection. Stenoses distal to the anastomosis were bridged by neovascularization. This was similar to the neovascularization observed by the authors in rat hearts injected with FGF-1. Histological sections of rat myocardium showed a threefold increase in microvessel density. In 20 patients undergoing similar coronary artery bypass surgery in whom inactivated FGF-1 was injected, there was no

evidence of myocardial neovascularization on the 12-week angiogram.

An advantage of this approach is that it induces local angiogenesis and appears to avoid high levels of circulating angiogenic activity that could possibly stimulate plaque angiogenesis and secondary plaque growth. Why does neovascularization persist for at least 12 weeks after only a single set of injections of the angiogenic protein? Perhaps intramyocardial injections of the angiogenic protein? Perhaps persistent neovascularization was facilitated by upregulation of VEGF and its receptors in hypoxic tissue.[18] Furthermore, basic FGF and VEGF are synergistic mitogens for endothelial cells in vitro.[19,20] Also, FGF can increase expression of (or mobilize) VEGF.[21]

This report uses primarily anatomic studies to demonstrate increased myocardial neovascularization after angiogenic therapy. We look forward to the follow-up of these patients to learn whether they have significant functional improvement compared with the control group of patients who received inactive FGF. It may be difficult to discriminate the extent to which functional improvement is due to the angiogenic therapy per se, despite use of a control group, because of the concomitant internal mammary artery anastomosis and the relatively small number of patients in this study. Nevertheless, the angiographic documentation of myocardial revascularization suggests that functional improvement should follow.

Although major therapeutic advances in cardiology have been based on the general principles of control of blood pressure, regulation of cardiac rhythm, enhancement of myocardial contractile strength, increased diameter of narrowed coronary arteries, and lysis of intravascular thromboses, the report by Schumacher et al introduces a new modality, the regulation of blood vessel growth. If angiogenic therapy of the myocardium continues to live up to its potential as indicated by this report, we may witness novel refinements in future years as the molecular biology of endothelial cell and smooth cell growth is gradually uncovered. For example, the therapeutic induction of coronary arterial collaterals may someday be optimized by administration of appropriate mixtures of molecules that target different components of the vasculature, ie, the FGFs are mitogenic for vascular endothelial cells and smooth muscle, VEGF[22] is mitogenic primarily for endothelial cells, angiopoietin-1 mediates the recruitment of smooth muscle cells to the wall of new vessels,[23] and angiopoietin-2 appears to prevent or downregulate smooth muscle apposition to the walls of microvessels.[24] It is interesting that the methodology to discover these different vascular cell growth proteins emerged largely from investigations of mechanisms of tumor angiogenesis in studies funded primarily by the National Cancer Institute over many years. The report by Schumacher et al illustrates how unpredictable are the clinical applications that may arise from basic research in a different field.

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Growing New Blood Vessels with a Timed-Release Capsule of Growth Factor is a Promising Treatment for Heart Bypass Patients, Finds NHLBI Study

By The National Heart, Lung, and Blood Institute

Heart bypass patients treated with a timed-release capsule of a substance that promotes the growth of new blood vessels showed evidence of improved blood supply and heart function, according to a study supported by the National Heart, Lung, and Blood Institute (NHLBI) of the National Institutes of Health.

"Growing" blood vessels, a strategy called angiogenesis, is a promising experimental treatment for blocked arteries in bypass surgery patients for whom surgery alone would not adequately restore blood flow to the heart.

Dr. Michael Simons and colleagues at Harvard Medical School inserted timed-release capsules of basic fibroblast growth factor (bfGF) into the heart muscle of patients scheduled for bypass surgery. Patients received either a 10 microgram (mcg) or 100 mcg dose

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of the substance. Other patients received a harmless placebo capsule at the time of surgery. The relatively small study (24 patients total) was designed to test the safety and effectiveness of the procedure.

The study, published in the November 2, 1999 issue of *Circulation*, found that there were no serious adverse effects of the treatment. Both magnetic resonance imaging (MRI) and nuclear stress testing were used to evaluate changes in blood flow. Stress tests showed a worsening of blood flow in the placebo group, no change in the 10 mcg. group and significant improvement in patients receiving 100 mcg. MRI results showed clear improvement in blood flow in patients given 100 mcg. Patients in the highest dose group were free of angina (chest pain) but some patients in the placebo and low-dose group experienced chest pain.

Simons and colleagues note that a larger (Phase II) multi-center study of this approach is currently underway.

The National Heart, Lung, and Blood Institute of The National Institutes of Health. Press Release: **Growing New Blood Vessels with a Timed-Release Capsule of Growth Factor Is a Promising Treatment for Heart Bypass Patients, Finds NHLBI Study**. November 1, 1999. (Online) http://www.nih.gov/news/pr/nov99/nhlbi-01.htm

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(1 of 1)

United States Patent	5,652,225
Isner	**July 29, 1997**

Methods and products for nucleic acid delivery

Abstract

The present invention provides a method for the delivery of a nucleic acid to an arterial cell comprising contacting the cell with a hydrophilic polymer incorporating the nucleic acid. The nucleic acid may be any nucleic acid, including antisense DNA or RNA. The nucleic acid may encode hormones, enzymes, receptors or drugs of interest. The nucleic acid is selected based upon the desired therapeutic outcome. For example, in the treatment of ischemic diseases, one would select a DNA encoding an angiogenic protein. The nucleic acid may be carried by a microdelivery vehicle such as cationic liposomes and adenoviral vectors. DNA encoding different proteins may be used separately or simultaneously.

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Appl. No.: **675523**
Filed: **July 3, 1996**

U.S. Class:	514/44; 604/51; 604/52; 604/53; 536/23.5; 536/23.51; 435/320.1; 435/172.1; 435/172.3; 935/9; 935/22; 935/32; 935/33; 935/34; 935/52; 935/57; 424/93.2
Intern'l Class:	A01N 047/40
Field of Search:	514/44 604/51,52,53 536/23.5,23.51 435/320.1,172.1,172.3,235.1,240.2 935/9,22,32,33,34,52,57 424/93.2

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Parent Case Text

This is a continuation of application Ser. No. 08/318,045 filed on Oct. 4, 1994 now abandoned.

Claims

1. A method for inducing the formation of new blood vessels in a desired target tissue in a human host, comprising contacting an arterial cell in an artery or blood vessel via a balloon catheter coated with a hydrogel polymer admixed with a first DNA encoding an angiogenic protein selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor .alpha. and .beta., platelet-derived endothelial

growth factor, platelet-derived growth factor, tumor necrosis factor .alpha., hepatocyte growth factor and insulin like growth factor and having an operably linked secretory signal sequence or a first DNA encoding a modified angiogenic protein selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor .alpha. and .beta., platelet-derived endothelial growth factor, platelet-derived growth factor, tumor necrosis factor .alpha., hepatocyte growth factor and insulin like growth factor having an operably linked secretory signal sequence, wherein said anglogenic protein induces new blood vessel formation when expressed in said target tissue in an amount effective to induce new blood vessel formation.

2. The method of claim 1, wherein the angiogenic protein is vascular endothelial growth factor.

3. The method of claim 1, wherein the hydrogel polymer is selected from the group consisting of polycarboxylic acids, cellulosic polymers, gelatin, polyvinylpyrrolidone, maleic anhydride polymers, polyamides, polyvinyl alcohols, and polyethylene oxides.

4. The method of claim 1, wherein the hydrogel polymer is a polyacrylic acid polymer.

5. The method of claim 1, wherein the hydrogel polymer is admixed with a second DNA encoding an angiogenic protein selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor .alpha. and .beta., platelet-derived endothelial growth factor, platelet-derived growth factor, tumor necrosis factor .alpha., hepatocyte growth factor and insulin like growth factor and having an operably linked secretory signal sequence or a second DNA encoding a modified angiogenic protein selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor .alpha. and .beta., platelet-derived endothelial growth factor, platelet-derived growth factor, tumor necrosis factor .alpha., hepatocyte growth factor and insulin like growth factor having an operably linked secretory signal sequence, wherein said angiogenic protein induces new blood vessel formation when expressed in said target tissue in an amount effective to induce new blood vessel formation, and wherein said second DNA is not the same as said first DNA.

6. A method for inducing the formation of new blood vessels in a desired target tissue in a human host, comprising contacting an arterial cell in an artery or blood vessel via a balloon catheter coated with a hydrogel polymer admixed with DNA encoding vascular endothelial growth factor and which is expressed in an amount effective to induce new blood vessel formation.

Description

FIELD OF THE INVENTION

The present invention relates to delivery of nucleic acid to arterial cells and compositions therefor.

BACKGROUND OF THE INVENTION

Work from several laboratories (Nabel, et al., Science, 249:1285-1288 (1990); Lim, et al., Circulation, 83:2007-2011 (1991); Flugelman, et al., Circulation, 85:1110-1117 (1992); Leclerc, et al., J. Clin. Invest., 90:936-944 (1992); Chapman, et al., Circ. Res., 71: 27-33 (1992); Riessen, et al., Hum. Gene Ther., 4: 749-758 (1993); and Takeshita, et al., J. Clin. Invest., 93:652-661 (1994), has demonstrated

that recombinant marker genes could be transferred to the vasculature of live animals.

Gene delivery systems employed to date have been characterized by two principal components: a macrodelivery device designed to deliver the DNA/carrier mixture to the appropriate segment of the vessel, and microdelivery vehicles, such as liposomes, utilized to promote transmembrane entry of DNA into the cells of the arterial wall. Macrodelivery has typically been achieved using one of two catheters initially developed for local drug delivery: a double-balloon catheter, intended to localize a serum-free arterial segment into which the carrier/DNA mixture can be injected, or a porous-balloon catheter, designed to inject gene solutions into the arterial wall under pressure. Jorgensen et al., Lancet 1:1106-1108, (1989); Wolinsky, et al., J. Am. Coil. Cardiol., 15:475-485 (1990); March et al., Cardio Intervention, 2:11-26 (1992)); WO93/00051 and WO93/00052.

Double balloon catheters are catheters which have balloons which, when inflated within an artery, leave a space between the balloons. The prior efforts have involved infusing DNA-containing material between the balloons, allowing the DNA material to sit for a period of time to allow transfer to the cells, and then deflating the balloons, allowing the remaining genetic material to flush down the artery. Perforated balloons are balloons which have small holes in them, typically formed by lasers. In use, fluid containing the genetic material is expelled through the holes in the balloons and into contact with the endothelial cells in the artery. These gene delivery systems however, have been compromised by issues relating to efficacy and/or safety.

Certain liabilities, however, inherent in the use of double-balloon and porous balloon catheters have been identified. For example, neither double-balloon nor porous balloon catheters can be used to perform the angioplasty itself. Thus, in those applications requiring both angioplasty and drug delivery, e.g., to inhibit restenosis, two procedures must be preformed. Additionally, the double balloon typically requires long incubation times of 20-30 min., while the high-velocity jets responsible for transmural drug delivery from the porous balloon catheter have been associated with arterial perforation and/or extensive inflammatory infiltration (Wolinsky, et al., J. Am. Coil. Cardiol., 15:475-481 (1990).

SUMMARY OF THE INVENTION

It has now been discovered that nucleic acids can be delivered to cells of an artery or blood vessel by contacting the cells with a hydrophilic polymer incorporating the nucleic acid, thus avoiding the use of a double-balloon or porous balloon catheter and the problems associated with such delivery systems. It has also been demonstrated that, unexpectedly, the percentage of transduced arterial cells is significantly higher using the present invention compared with use of a double-balloon catheter.

By "arterial cells" is meant the cells commonly found in mammalian arteries, including endothelial cells, smooth muscle cells, connective tissue cells and other cells commonly found in the arterial structure.

By "nucleic acid" is meant DNA and RNA, including antisense DNA or RNA.

It has further been discovered that a DNA encoding an angiogenic protein (a protein capable of inducing angiogenesis, i.e., the formation of new blood vessels), delivered by the method of the present invention is expressed by the arterial cell and induces angiogenesis in tissues perfused by the treated blood vessels. This allows for the treatment of diseases associated with vascular occlusion in a variety of target tissues, such as limb ischemia, ischemic cardiomyopathy, myocardial ischemia, cerebral ischemia and portal hypertension.

The present invention provides a method for the delivery of a nucleic acid to an arterial cell comprising contacting the cell with a hydrophilic polymer incorporating the nucleic acid. The nucleic acid may be any nucleic acid, DNA and RNA, including antisense DNA or RNA. The DNA may encode hormones, enzymes, receptors or drugs of interest. The nucleic acid is selected based upon the desired therapeutic outcome. For example, in the treatment of ischemic diseases, the genetic material of choice is DNA encoding an angiogenic protein. The nucleic acid may be carried by a microdelivery vehicle such as cationic liposomes and adenoviral vectors. DNA encoding different proteins may be used separately or simultaneously.

The hydrophilic polymer is selected to allow incorporation of the nucleic acid to be delivered to the arterial cell and its release when the hydrophilic polymer contacts the arterial cell. Preferably, the hydrophilic polymer is a hydrogel polymer. Other hydrophilic polymers will work, so long as they can retain the genetic material of the present invention, so that, on contact with arterial cells, transfer of genetic material occurs.

Suitable hydrogel polymers include, for example, those selected from the group consisting of polycarboxylic acids, cellulosic polymers, gelatin, polyvinylpyrrolidone, maleic anhydride polymers, polyamides, polyvinyl alcohols, and polyethylene oxides. The hydrogel polymer is preferably polyacrylic acid.

Without wishing to be bound by theory, one reason that the use of hydrogel, and particularly with hydrogel coated balloon catheters, is believed to provide improved results over, for example, prior treatments with double balloon catheters, is that the use of standard balloon catheters with hydrogel surfaces causes the hydrogel not only to contact the endothelial cells which line the interior of the arteries, but also displaces the endothelial cells sufficiently to permit contact between the hydrogel and the smooth muscle cells which underlie the endothelial cell layer. This permits expression of polypeptides in different arterial cell types, which enhances the kinds and amounts of therapeutic polypeptides which can be produced in accordance with this invention. For example, as indicated in the examples below, the present method successfully produces sufficient amounts of vascular endothelial growth factor (VEGF) to cause angiogenesis downstream from a DNA/arterial contact point, despite the fact that VEGF is not normally produced even by transformed endothelial cells, but is produced by smooth muscle cells of the type that surround the endothelial cells in the artery.

The arterial cell may be contacted with the hydrophilic polymer incorporating the DNA by means of an applicator such as a catheter which is coated with the DNA-bearing hydrophilic polymer. Preferably, the applicator can exert some pressure against the arterial cells, to improve contact between the nucleic acid-bearing hydrophilic polymer and the arterial cells. Thus a balloon catheter is preferred. Preferably, the hydrophilic polymer coats at least a portion of an inflatable balloon of the balloon catheter.

The present invention further includes compositions comprising hydrophilic polymers incorporating nucleic acid. Preferably the hydrophilic polymer is a hydrogel and the nucleic acid is DNA which encodes an angiogenic protein.

The present invention also provides kits for application of genetic material to the interior of an artery or similar bodily cavity, comprising a substrate, such as a catheter or a suitably shaped rod, and a source of genetic material comprising the DNA coding for the desired therapeutic polypeptide. Preferably, the present invention is directed to a catheter adapted for insertion into a blood vessel, having a balloon element adapted to be inserted into the vessel and expandable against the walls of the

vessel. At least a portion of the balloon element is defined by a coating of a hydrophilic polymer, and incorporated within the hydrophilic polymer coating, a nucleic acid to be delivered to the arterial cell. The hydrophilic polymer is preferably a hydrogel polymer, most preferably a hydrophilic polyacrylic acid polymer.

The present invention also provides a method for inducing angiogenesis in a desired target tissue, comprising delivering a DNA encoding an angiogenic protein to an arterial cell in an artery or blood vessel perfusing the target tissue.

Other aspects of the invention are discussed infra.

As used herein the term "angiogenic protein" means any protein, polypeptide, mutein or portion thereof that is capable of inducing the formation of new blood vessels. Such proteins include, for example, acidic and basic fibroblast growth factors (aFGF and bFGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor .alpha. and .beta. (TGF-.alpha. and TFG-.beta.), platelet-derived endothelial growth factor (PD-ECGF), platelet-derived growth factor (PDGF), tumor necrosis factor .alpha. (TNF-.alpha.), hepatocyte growth factor (HGF) and insulin like growth factor. Preferably, the angiogenic protein contains a secretory signal sequence allowing for secretion of the protein from the arterial cell. VEGF is a preferred protein.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1(a) and 1(b) show the rabbit ischemic hindlimb model. FIG. 1(a)is a representative angiogram recorded 10 days after surgery. Once the femoral artery is excised, thrombotic occlusion of the external lilac artery extends retrograde to its origin from the common lilac (arrow). Open arrow indicates the site of arterial gene transfer. In FIG. 1(b) the shaded segment of femoral artery has been excised.

FIGS. 2(a), 2(b) and 2(c) illustrate (a) RT-PCR analysis of transfected arteries, (b) Southern blot analysis of RT-PCR products and (c) nucleotide sequence of the RT-PCR product from transfected rabbit lilac artery. In FIGS. 2(a) and 2(b) the expression of the human VEGF mRNA was evident in the rabbit lilac artery (lane 4) and cultured rabbit vascular smooth muscle cells (lane 6, positive control) which were transfected with human VEGF gene. Arrows indicate position of VEGF band at 258 bp. Lane 1 depicts the results using a molecular weight marker, namely pGEM3zf(-) digested with Hae III; lane 2 is a negative control (no RNA); lane 3 is a second negative control (rabbit lilac artery transfected with .beta.-galactosidase expression plasmid); and lane 5 is a further negative control (PCR analysis of the VEGF-transfected lilac artery excluding the reverse transcriptase reaction). FIG. 2(c) shows the nucleotide sequence of the RT-PCR product from a transfected rabbit lilac artery. Direct sequencing of the 258 bp bands obtained by RT-PCR confirmed that this band represented the human VEGF sequence. The sequence designated in 2(c) corresponds to amino acids 69 to 75 of the VEGF peptide. Asterisks denote the nucleotides which are not conserved among different species of the VEGF gene (rat, mouse, bovine, guinea pig) demonstrating that the exogenous human gene was amplified by the RT-PCR procedure.

FIGS. 3A, 3B, 3C, 3D, 3E and 3F comprise internal lilac angiography of a control rabbit at (A) day 0 (pre-transfection), (B) day 10, and (C) day 30 post-transfection, and of a VEGF-transfected rabbit at (D) day 0, (E) day 10, and (F) day 30 post-transfection. In contrast to the control, angiographic examination of the VEGF-transfected animal discloses extensive collateral artery formation.

FIGS. 4(a), 4(b) and 4(c) are graphs illustrating the effect of VEGF-transfection on revascularization

in an ischemic limb model. FIG. 4(a) the angiographic score at day 0 (immediately prior to transfection), and days 10 and 30 post-transfection. FIG. 4(b) Calf Blood pressure ratio at day 0, and at days 10 and 30 post-transfection. FIG. 4(c) depicts capillary density at day 30 post-transfection. (*p<0.05,**p<0.01)

FIGS. 5(a) and 5(b) illustrate alkaline phosphatase staining of ischemic hindlimb muscle, counterstained with eosin. FIG. 5(a) depicts the muscle of an animal transfected with pGSVLacZ. FIG. 5(b) depicts the muscle of an animal transfected with phVEGF.sub.165. The dark staining indicates capillaries as shown by the arrows.

FIG. 6 illustrates a diagrammatical cross section of a balloon catheter having a hydrophilic surface bearing genetic material in accordance with the present invention, in place within an artery.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for the delivery of nucleic acid to an arterial cell comprising contacting the cell with a hydrophilic polymer incorporating the nucleic acid.

The nucleic acid may be any nucleic acid which when introduced to the arterial cells provides a therapeutic effect. The nucleic acid is selected based upon the desired therapeutic outcome. For example, in the treatment of ischemic diseases, one genetic material of choice would be a DNA encoding an angiogenic protein. DNA useful in the present invention include those that encode hormones, enzymes, receptors or drugs of interest. The DNA can include genes encoding polypeptides either absent, produced in diminished quantities, or produced in mutant form in individuals suffering from a genetic disease. Additionally it is of interest to use DNA encoding polypeptides for secretion from the target cell so as to provide for a systemic effect by the protein encoded by the DNA. Specific DNA's of interest include those encoding hemoglobin, interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-4, interleukin-5, interleukin-6, interleukin-7, interleukin-8, interleukin-9, interleukin-10, interleukin-11, etc., GM-CSF, G-CSF, M-CSR, human growth factor, insulin, factor VIII, factor IX, tPA, LDL receptors, tumor necrosis factor, PDGF, EGF, NGF, IL-1ra, EPO, .beta.-globin and the like, as well as biologically active muteins of these proteins. The nucleic acid utilized may also be "anti-sense" DNA or RNA, which binds to DNA or RNA and blocks the production of harmful molecules. In addition, the DNA carried to the arterial cells in accordance with the present invention may code for polypeptides which prevent the replication of harmful viruses or block the production of smooth muscle cells in arterial walls to prevent restenosis.

Antisense RNA molecules are known to be useful for regulating translation within the cell. Antisense RNA molecules can be produced from the corresponding gene sequences. The antisense molecules can be used as a therapeutic to regulate gene expression associated with a particular disease.

The antisense molecules are obtained from a nucleotide sequence by reversing the orientation of the coding region with regard to the promoter. Thus, the antisense RNA is complementary to the corresponding mRNA. For a review of antisense design see Green, et al., Ann. Rev. Biochem. 55:569-597 (1986), which is hereby incorporated by reference. The antisense sequences can contain modified sugar phosphate backbones to increase stability and make them less sensitive to RNase activity. Examples of the modifications are described by Rossi, et al., Pharmacol, Ther. 50(2):245-354, (1991).

In certain therapeutic applications, such as in the treatment of ischemic diseases, it may be desirable to induce angiogenesis, i.e., the formation of new blood vessels. For such applications, DNA's encoding growth factors, polypeptides or proteins, capable of inducing angiogenesis are selected. Folkman, et

al., Science, 235:442-447 (1987). These include, for example, acidic and basic fibroblast growth factors (aFGF and bFGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor .alpha. and .beta. (TGF-.beta. and TGF-.beta.), platelet-derived endothelial cell growth factor (PD-ECGF), platelet-derived growth factor (PDGF) itself, tumor necrosis factor .alpha. (TNF-.alpha.), hepatocyte growth factor (HGF) and insulin like growth factor. See, Klagsbrun, et al., Annu. Rev. Physiol., 53:217-239 (1991) and Folkman, et al., J. Biol. Chem. 267:10931-10934 (1992). Muteins or fragments of an angiogenic protein may be used as long as they induce or promote the formation of new blood vessels.

Recent investigations have established the feasibility of using recombinant formulations of such angiogenic growth factors to expedite and/or augment collateral artery development in animal models of myocardial and hindlimb ischemia. See, Baffour, et al., J. Vasc. Surg., 16:181-191 (1992) (bFGF); Pu, et al, Circulation, 88:208-215 (1993) (aFGF); Yanagisawa-Miwa, et al., Science, 257:1401-1403 (1992) (bFGF); Ferrara, et al., Biochem. Biophys. Res. Commun., 161:851-855 (1989) (VEGF).

VEGF was also purified independently as a tumor-secreted factor that included vascular permeability by the Miles assay (Keck, et al, Science, 246:1309-1342 (1989) and Connolly, et al., J. Biol Chem., 264:20017-20024 (1989)), and thus its alternate designation, vascular permeability factor (VPF). VEGF is a preferred angiogenic protein. Two features distinguish VEGF from other heparin-binding, angiogenic growth factors. First, the NH.sub.2 terminus of VEGF is preceded by a typical signal sequence; therefore, unlike bFGF, VEGF can be secreted by intact cells. Second, its high-affinity binding sites, shown to include the tyrosine kinase receptors Flt-1 and Flt-1/KDR are present on endothelial cells. Ferrara, et al., Biochem. Biophys. Res. Commun., 161:851-855 (1989) and Conn, et al., Proc. Natl. Acad. Sci. USA, 87:1323-1327 (1990). (Interaction of VEGF with lower affinity binding sites has been shown to induce mononuclear phagocyte chemotaxis). Shen, et al., Blood, 81:2767-2773 (1993) and Clauss, et al., J. Exp. Med., 172:1535-1545 (1990).

Evidence that VEGF stimulates angiogenesis in vivo had been developed in experiments performed on rat and rabbit cornea (Levy, et al., Growth Factors, 2:9-19 (1989) and Connolly, et al., J. Clin. Invest., 84:1470-1478 (1989)), the chorioallantoic membrane (Ferrara, et al., Biochem Biophys Res Commun., 161:851-855 (1989)), and the rabbit bone graft model. Connolly, et al., J. Clin. Invest., 84:1470-1478 (1989).

Preferably, the angiogenic protein contains a secretory signal sequence that facilitates secretion of the protein from the arterial cell. Angiogenic proteins having native signal sequences, e.g., VEGF, are preferred. Angiogenic proteins that do not have native signal sequences, e.g., bFGF, can be modified to contain such sequences using routine genetic manipulation techniques. See, Nabel et al., Nature 362:844 (1993).

The nucleotide sequence of numerous peptides and proteins, including angiogenic proteins, are readily available through a number of computer data bases, for example, GenBank, EMBL and Swiss-Prot. Using this information, a DNA segment encoding the desired may be chemically synthesized or, alternatively, such a DNA segment may be obtained using routine procedures in the art, e.g, PCR amplification.

To simplify the manipulation and handling of the DNA, prior to introduction to the arterial cell, the DNA is preferably inserted into a vector, e.g., a plasmid vector such as pUC118, pBR322, or other known plasmid vectors, that includes, for example, an E. Coli origin of replication. See, Sambrook, et al., Molecular Cloning:A Laboratory Manual, Cold Spring Harbor Laboratory press, (1989). The plasmid vector may also include a selectable marker such as the .beta.-lactamase gene for ampicillin

resistance, provided that the marker polypeptide does not adversely effect the metabolism of the organism being treated. Additionally, if necessary, the DNA may be operably linked to a promoter/enhancer region capable of driving expression of the protein in the arterial cell. An example of a suitable promoter is the 763-base-pair cytomegalovirus (CMV) promoter. Normally, an enhancer is not necessary when the CMV promoter is used. The RSV and MMT promoters may also be used. Certain proteins can expressed using their native promoter.

If desired, the DNA may be used with a microdelivery vehicle such as cationic liposomes and adenoviral vectors. For a review of the procedures for liposome preparation, targeting and delivery of contents, see Mannino and Gould-Fogerite, Bio Techniques, 6:682 (1988). See also, Felgner and Holm, Bethesda Res. Lab. Focus, 11(2):21 (1989) and Maurer, R. A., Bethesda Res. Lab. Focus, 11 (2):25 (1989). Replication-defective recombinant adenoviral vectors, can be produced in accordance with known techniques. See, Quantin, et al., Proc. Natl. Acad. Sci. USA, 89:2581-2584 (1992); Stratford-Perricadet, et al., J. Clin. Invest., 90:626-630 (1992); and Rosenfeld, et al., Cell, 68:143-155 (1992).

In certain situations, it may be desirable to use DNA's encoding two or more different proteins in order optimize the therapeutic outcome. For example, DNA encoding two angiogenic proteins, e.g., VEGF and bFGF, can be used, and provides an improvement over the use of bFGF alone. Or an angiogenic factor can be combined with other genes or their encoded gene products to enhance the activity of targeted cells, while simultaneously inducing angiogenesis, including, for example, nitric oxide synthase, L-argine, fibronectin, urokinase, plasminogen activator and heparin.

The hydrophilic polymer is selected to allow incorporation of the DNA to be delivered to the arterial cell and its release when the hydrophilic polymer contacts the arterial cell.

Preferably, the hydrophilic polymer is a hydrogel polymer, a cross-linked polymer material formed from the combination of a colloid and water. Cross-linking reduces solubility and produces a jelly-like polymer that is characterized by the ability to swell and absorb liquid, e.g., that containing the DNA. Suitable hydrogel polymers include, for example, those selected from the group consisting of polycarboxylic acids, cellulosic polymers, gelatin, polyvinylpyrrolidone, maleic anhydride polymers, polyamides, polyvinyl alcohols, and polyethylene oxides. Preferred hydrogels are polyacrylic acid polymers available as HYDROPLUS (Mansfield Boston Scientific Corp., Watertown, Mass.) and described in U.S. Pat. No. 5,091,205.

The nucleic acid in aqueous solution is incorporated into the hydrophilic polymer to form a nucleic acid-hydrophilic polymer composition. The nucleic acid is incorporated without complexing or chemical reaction with the hydrophilic polymer, and is preferably relatively freely released therefrom when placed in contact with the arterial cells. The resulting structure comprises a support, e.g. the balloon of the balloon catheter, on which is mounted the hydrogel, in or on which is incorporated the desired DNA and its associated vehicle, e.g., phage or plasmid vector. The hydrophilic polymer is preferably adhered to the support, so that after application of the DNA to the target cells, the hydrophilic polymer is removed with the support.

An arterial cell is contacted with the nucleic acid-hydrophilic polymer composition by any means familiar to the skilled artisan. The preferred means is a balloon catheter having the hydrophilic polymer on its outer surface, which permits the contact between the hydrophilic polymer bearing the nucleic acid to be transferred and the arterial cells to be made with some pressure, thus facilitating the transfer of the nucleic acid to the cells. However, other supports for the hydrophilic polymer are also useful, such as catheters or solid rods having a surface of hydrophilic polymer. Preferably, the catheters or

rods or other substrates which are flexible, to facilitate threading through the arteries to reach the point of intended application. For cells that are not in tubular arteries, other types of catheters, rods or needles may be used.

When a hydrophillic arterial balloon is used, it is not necessary to protect the balloon prior to inflation, since relatively little of the nucleic acid is lost in transit to the treatment site until the balloon is inflated and the hydrophilic polymer bearing the nucleic acid is pressed against the arterial cells. When hydrophilic polymer-surfaced catheters or rods are used as the vehicle or substrate, the surface can protected, e.g. by a sheath, until the point of intended application is reached, and then the protection removed to permit the hydrophilic polymer bearing the nucleic acid to contact the arterial cells.

The vehicle, be it arterial balloon, catheter, flexible rod or other shaped vehicle, can be furnished with means to assist in accurate placement within the intended body cavity. For example, it can be furnished with a radioactive element, or made radio-opaque, furnished with means permitting easy location using ultrasound, etc.

Preferably, the nucleic acid-hydrophilic composition contacts the arterial cell by means of a catheter. The catheter is preferably a balloon catheter constructed for insertion in a blood vessel and has a catheter shaft and an expandable dilation balloon mounted on the catheter shaft. At least a portion of the exterior surface of the expandable portion is defined by a coating of a tenaciously adhered hydrophilic. Incorporated in the hydrophilic polymer is an aqueous solution of the DNA to be delivered to the arterial cells.

In general, when dry, the hydrophilic polymer (preferably hydrogel) coating is preferably on the order of about 1 to 10 microns thick, with a 2 to 5 micron coating typical. Very thin hydrogel coatings, e.g., of about 0.2-0.3 microns (dry) and much thicker hydrogel coatings, e.g., more than 10 microns (dry), are also possible. Typically, hydrogel coating thickness may swell by about a factor of 2 to 10 or more when the hydrogel coating is hydrated.

Procedures for preparing a balloon with a hydrogel coating are set forth in U.S. Pat. No. 5,304,121, the disclosure of which is incorporated herein by reference.

A representative catheter is set forth in FIG. 6. Referring to FIG. 6, 1 is the wall of the blood vessel. The figure shows the catheter body 2 held in place by the inflation of an inflation balloon 3. The balloon comprises a hydrogel coating 4 incorporating DNA 5.

In use, the DNA, for example, is applied ex vivo to the hydrophilic polymer coating of the balloon. To facilitate application, the balloon may be inflated. If necessary, the polymer may be dried with warm air and the DNA application repeated. The amount of DNA to be applied to the arterial surface depends on the purpose of the DNA and the ability of the DNA to be expressed in the arterial cells. Generally, the amount of naked DNA applied to the balloon catheter is between about 0.1 and 100 .mu.g/mm.sup.2, more preferably between about 0.5 and about 20 .mu.g/mm.sup.2, most preferably between about 1.5 and about 8 .mu.g/mm.sup.2. Preferably, between 0.5 mg and 5 mg of DNA are applied to the hydrogel coating of a balloon catheter having an inflated lateral area of about 630 mm.sup.2 (e.g., a balloon catheter having an inflated diameter of about 5 mm and a length of about 40 mm), providing a surface having about 0.8 to about 8 .mu.g/mm.sup.2 of DNA when the balloon is inflated and contacts the interior of the artery. More preferably, between 1 mg and 3 mg of DNA are applied to the polymer, providing a DNA loading of about 1.6 to about 4.8 .mu.g/mm.sup.2.

The catheter is inserted using standard percutaneous application techniques and directed to the desired location, e.g., an artery perfusing the target tissue. For example, in the treatment of patients with occlusive peripheral arterial disease (PAD), the balloon is directed towards an artery of the leg, e.g., lilac. Once the balloon has reached its desired location, it is inflated such that the hydrogel coating of the balloon contacts the arterial cells located on the walls of the artery and remains inflated for a time sufficient to allow transfer of the DNA encoding the angiogenic protein from the hydrogel to the arterial cells. Preferred periods of balloon inflation range from 30 seconds to 30 minutes, more preferably 1 minute to 5 minutes. Surprisingly, that is normally sufficient time to permit transfer of the DNA by the method of the present invention.

Once transferred, the DNA coding for the desired therapeutic polypeptide is expressed by the arterial cells for a period of time sufficient for treatment of the condition of interest. Because the vectors containing the DNA of interest are not normally incorporated into the genome of the cells, however, expression of the protein of interest takes place for only a limited time. Typically, the therapeutic protein is only expressed in therapeutic levels for about two days to several weeks, preferably for about 1-2 weeks. Reapplication of the DNA can be utilized to provide additional periods of expression of the therapeutic polypeptide. If desired, use of a retrovirus vector to incorporate the heterologous DNA into the genome of the arterial cells will increase the length of time during which the therapeutic polypeptide is expressed, from several weeks to indefinitely.

In one preferred application, the DNA-hydrogel polymer composition can be used to deliver a DNA encoding an angiogenic protein to an arterial cell in an artery or blood vessel perfusing the target tissue. Expression of the angiogenic protein and its secretion from the arterial cell induces angiogenesis, i.e., the formation of new blood vessels, in target tissues perfused by the artery or blood vessels, allowing for the treatment of diseases associated with vascular occlusion such as limb ischemia, ischemic cardiomyopathy, myocardial ischemia, cerebral ischemia and portal hypertension.

The present invention makes genetic treatment possible which can correct heretofore incorrectable problems.

The present invention is further illustrated by the following examples. These examples are provided to aid in the understanding of the invention and are not construed as a limitation thereof.

EXAMPLE 1

Direct Gene Transfer with Hydrogel Polymer Balloon Catheter Applied to an Angioplasty Catheter Balloon Can be Used to Effect Direct Gene Transfer to the Arterial Wall.

DNA solution was applied to the surface of an angioplasty catheter balloon with a hydrogel polymer (marketed under the mark Slider.TM. with Hydroplus.RTM. by Mansfield Boston Scientific Corp., Watertown, Mass.). The catheter was constructed with a single polyethylene balloon, 2.0 mm in diameter and 2.0 cm in length. The Hydroplus.RTM. coating consists of a hydrophilic polyacrylic acid polymer, crosslinked via an isocyanate onto the balloon to form an ultra-high molecular weight hydrogel with tight adherence to the balloon surface. The thickness of the hydrogel coating when dry measures between 3-5 .mu.m; upon exposure to an aqueous environment, the coating swells to 2-3 times its initially dry thickness. In order to apply DNA to the catheter, the balloon was inflated to 4 atm, following which 20 .mu.l of DNA solution were pipetted and distributed onto the balloon surface using a sterile pipette tip. After the balloon's hydrogel polymer was covered with a homogeneous film of DNA solution, the hydrogel was dried with warm air. The above procedure was then repeated, resulting in a total of 40 .mu.l of DNA solution applied to the balloon.

For percutaneous application, luciferase DNA concentration was 3.27 .mu.g/.mu.l. DNA was dissolved in TE buffer (10 mM Tris, 1 mM EDTA).

(Attempts were made to apply DNA solution to standard uncoated balloons as well. The hydrophobic surface of the polyethylene balloon, however, made it impossible to cover the balloon with a film of DNA solution.)

To determine the total amount of DNA which is successfully absorbed onto the balloon surface, 5 hydrogel balloons were coated with 40 .mu.l DNA (2 .mu.g DNA/.mu.l) containing a small amount of .sup.35 S-labeled luciferase plasmid. (Levy, et al., Growth Factors, 2:1535-1545 (1990)). A random primed DNA labeling kit (United States Biochemical, Cleveland, Ohio) was used for the labeling reaction an unincorporated nucleotides were remove by ethanol precipitation. After the coating procedure, the catheter tip was placed in 0.5 ml water for 15 minutes at room temperature, and 1.0 ml gel solubilizer (Solvable,.TM. New England Nuclear, Boston, Mass.) for 3 hours at 50.degree. C. to dissolve the gel before the scintillation fluid was added. The amount of DNA on the balloon was calculated from the quotient: [counts per minute (cpm) in a scintillation vial containing the balloon]/[cpm in a vial containing 40 .mu.l of the same lot of labeled DNA (80 .mu.g)]. Scintillation counts were corrected for quench and chemiluminescence.

After coating hydrogel balloons with 40 .mu.l of DNA solution (containing 80 .mu.g of radiolabeled DNA), and drying the gel, the magnitude of DNA retained on the hydrogel balloon was determined by comparing the amount of radioactivity on the balloons to the amount of radioactivity in 40 .mu.l of the original radiolabeled DNA solution. Scintillation counting revealed that 97.+-.2% (n=5) of the radioactively labeled DNA remained on the hydrogel coated balloon, corresponding to 78.+-.1.5 .mu.g of luciferase DNA.

Reporter Genes

The firefly luciferase gene and the gene for nuclear-specific .beta.-galactosidase (.beta.-gal) were used as reporter genes to monitor the results of the transfection procedures. The luciferase expression vector, pRSVLUC (courtesy of Dr. Allen Brasier, Massachusetts General Hospital, Boston, Mass.), consist of a full length Photinus pyralis luciferase cDNA (pJD 204) (de Wet et al., 1987) inserted into a PGEM3-plasmid (Brasier et al. Biotechniques, 7:1116-1122 (1989)), under the control of Rous sarcoma virus long terminal repeat (RSV-LTR) promoter. The pGSVLacZ vector contains the simian virus (SV40) large tumor nuclear location signal fused to the lacZ gene (nls .beta.-gal) (Bonnerot et al., Proc. Natl. Acad. Sci. U.S.A., 84:6795-6799 (1987)) (gift from Dr. Claire Bonnerot, Institut Pasteur, Paris, France), inserted into a pGEM1-plasmid. Nuclear staining identifies the exogenous construct designed to permit nuclear translocation, and thus distinguishes expression of the transgene from endogenous (cytoplasmic) .beta.-gal activity. Previous concerns (Lim et al., Circulation, 83:2007-2011 (1991)) regarding nonspecificity of blue staining resulting from .beta.-gal are thus eliminated.

Analysis of Luciferase Activity

The magnitude of gene expression was determined by measuring luciferase activity as described previously (Leclerc et al., J. Clin. Invest., 90:936-944 (1992)) using the Luciferase Assay System (Promega, Madison, Wis.). Briefly, frozen arteries were homogenized and dissolved in 300 .mu.l of Cell Culture Lysis Reagent (Promega) containing 1 mg/ml bovine serum albumin. Three different 20-.mu.l aliquots prepared from each transfected specimen were mixed in a sample tube with 100 .mu.l of

Luciferase Assay Reagent (Promega, Madison, Wis.) and inserted into a luminometer (Model 20e, Turner Design, Sunnyvale, Calif.) that reports results on a scale established to yield as low as $10.^{-3}$ Turner light units (TLU). The specimen's total luciferase activity was calculated from the mean of the three aliquots analyzed. The luciferase values were in the linear range of a standard curve derived from samples with a known amount of luciferase (Sigma, St. Louis, Mo., catalogue #L9009). The lyophilized luciferase was, according to the manufacturer's instructions, dissolved in sterile water and further diluted in Cell Culture Lysis Reagent with 1 mg/ml bovine serum albumin. The following equation was used to convert TLU into pg luciferase: Luciferase [pg]=-0.08+0.051 TLU. Using this formula, 100 TLU corresponds to 5.0 pg of luciferase. It must be noted that the specific activity of luciferase standards from different vendors can vary considerably (Wolff, et al., Biotechniques, 11:474-485 (1991)); therefore, direct comparisons of luciferase reported by different groups must be made with caution, especially when the origin of the standard used is not specified.

Percutaneous Transfection

Percutaneous gene transfer experiments with the luciferase gene were performed in 13 rabbits using a catheter with a balloon to which a 20 .mu.m hydrogel coating had been applied and which was advanced through a 5 F teflon sheath. The balloon was advanced beyond the distal tip of the sheath, coated with 130 .mu.g luciferase DNA, ánd pulled back into the sheath to protect the balloon from subsequent contact with blood. The sheath and the angioplasty catheter were then introduced via the right carotid artery and advanced to the left common lilac artery under fluoroscopic control. The balloon catheter was advanced 2 cm further (beyond the distal sheath tip) into the external lilac artery and inflated there for 1 or 5 min. Following balloon deflation, the catheter system was removed. In 10 animals, the transfected external lilac artery as well as the contralateral control artery were removed 3 days later, weighed, and assayed for luciferase activity. In 3 additional animals, which had been transfected for 5 min. only, the arteries were excised 14 days after gene transfer. In these 3 animals we also removed the left femoral artery to check for luciferase expression directly downstream of the transfected segment.

Results

Luciferase expression was detected in all 10 (100%) percutaneously transfected arteries excised after 3 days, whether inflated for 5 min (386.+-.299 TLU, n=5) or 1 min (113.+-.59 TLU, n=5).

Three additional animals, in which balloons were inflated for 5 min only, were sacrificed after 14 days. Individual luciferase expression was 152.6, and 16 TLU, respectively (mean=58.+-.47 TLU). In this series, we also measured luciferase in the adjacent femoral artery, which was not inflated. Luciferase expression in all these arteries was undistinguishable from background activity (mean 0.04.+-.0.29 TLU).

The findings demonstrate that endoluminal vascular gene transfer can be achieved successfully and consistently with pure DNA applied to a standard angioplasty catheter balloon coated with hydrogel polymer. The hydrogel provides the absorbable medium to which one may apply a solution of pure DNA. Drying of the gel results in a layer of concentrated DNA which is then transferred to the arterial wall as the balloon contacts the arterial wall coincident with balloon inflation. Experiments with radiolabeled DNA established that 97% of DNA applied in aqueous solution to the hydrogel-coated balloon was still present on the balloon after drying of the gel. Autoradiograms of the arterial wall demonstrated that inflation of the hydrogel balloon results in DNA uptake which is distributed across the full thickness of the arterial wall. DNA was shown to penetrate the intact internal elastic lamina and was distributed intracellularly as well as extracellularly.

Despite elimination of accessory transfection vehicles in this example, both the frequency of successful transfection and the magnitude of reporter gene expression achieved were superior to that previously reported from our laboratory (Leclerc, et al., J. Clin Invest., 90:936-944 (1992)) and comparable to the results achieved by others (Chapman, et al., Circ. Res., 71:27-33 (1992) and Lim, et al., Circulation, 83:2007-2011 (1991)) using alternative delivery schemes. The success rate of transfection in our rabbit model as measured by expression of the luciferase transgene was 100% (37 of 37 artery segments), even in those cases in which the inflation time was reduced to one minute. The duration of inflation within a range from 10 to 30 minutes did not have significant impact on transfection efficiency, a feature which would be expected to facilitate human arterial, particularly coronary, gene transfer.

EXAMPLE 2

Induction of Angiogeneses In Vivo

Methods

Animal Model (FIG. 1).

The angiogenic response to transfection of the gene for vascular endothelial growth factor (VEGF) was investigated using a rabbit ischemic hindlimb model. Takeshita, et al., J. Clin. Invest., 93:662-670 (1994) and Pu, et al., J. Invest Surg., (In Press). All protocols were approved by St. Elizabeth's Institutional Animal Care and Use Committee. Male New Zealand White rabbits weighing 4-4.5 kg (Pine Acre Rabbitry, Norton, Mass.) were anesthetized with a mixture of ketamine (50 mg/kg) and acepromazine (0.8 mg/kg) following premedication with xyazine (2.5 mg/kg). A longitudinal incision was then performed, extending inferiorly from the inguinal ligament to a point just proximal to the patella. The limb in which the incision was performed--right versus left--was determined at random at the time of surgery by the surgeon. Through this incision, using surgical loops, the femoral artery was dissected free along its entire length; all branches of the femoral artery, including the inferior epigastric, deep femoral, lateral circumflex and superficial epigastric arteries, were also dissected free. After further dissecting the popliteal and saphenous arteries distally, the external lilac artery as well as all of the above arteries were ligated. Finally, the femoral artery was completely excised from its proximal origin as a branch of the external lilac artery, to the point distally where it bifurcates into the saphenous and popliteal arteries. Once the femoral artery is excised, thrombotic occlusion of the external lilac artery extends retrograde to its origin from the common lilac (FIG. 1(a), arrow). As a result, the blood supply to the distal limb is dependent on the collateral arteries which may originate from the internal lilac artery. Accordingly, direct arterial gene transfer of VEGF was performed in to the internal lilac artery of the ischemic limb. Post-operatively, all animals were closely monitored. Analgesia (levorphanol tartrate 60 mg/kg, Roche Laboratories, Nutley, N.J.) was administered subcutaneously as required for evidence of discomfort throughout the duration of the experiment. Prophylactic antibiotics (enrofloxacin 2.5 mg/kg, Miles, Shawnee Mission, Kans.) was also administered subcutaneously for a total of 5 days post-operatively.

Plasmids and Smooth Muscle Cell (SMC) Transfection in Vitro.

Complementary DNA clones for recombinant human VEGF.sub.165, isolated from cDNA libraries prepared from HL60 leukemia cells, were assembled into a mammalian expression vector containing the cytomegalovirus promoter. Leung, et al., Science, 246:1306-1309 (1989). The biological activity of VEGF.sub.165 secreted from cells transfected with this construct (phVEGF.sub.165) was

previously confirmed by the evidence that media conditioned by transfected human 293 cells promoted the proliferation of capillary cells. Leung, et al., Science, 246:1306-1309 (1989).

To evaluate expression of phVEGF.sub.165 in vascular cells, rabbit arterial smooth muscle cells (SMCs) were transfected in vitro. Cells were cultured by explant outgrowth from the thoracic aorta of New Zealand White rabbits. The identity of vascular SMCs was confirmed morphologically using phase contrast microscopy and by positive immunostaining using a monoclonal antibody to smooth muscle .alpha.-actin (Clone 1A4, Sigma, St. Louis, Mo.). Cells were grown in the media (M199, GIBCO BRL, Gaithersberg, Md.) supplemented with 10% fetal bovine serum (FBD, GIBCO BRL). In vitro transfection was performed by incubating SMCs (1.48.times.10.sup.6 cells/10 cm plate) with 11.5 .mu.g of the plasmid DNA and 70 .mu.g of liposomes (Transfection-reagent, Boehringer Mannheim, Indianapolis, Ind.) as previously described. Pickering, et al., Circulation, 89:13-21 (1994). After completion of transfection, media was changed to 10% FBS. Culture supernatant was sampled at 3 days pot-transfection, and was analyzed by ELISA assay for VEGF protein. Houck, et al., J. Biol. Chem. 267:26031-26037 (1992).

The plasmid pGSVLacZ (courtesy of Dr. Claire Bonnerot) containing a nuclear targeted .beta.-galactosidase sequence coupled to the simian virus 40 early promoter (Bonnerot, et al., Proc. Natl. Acad. Sci. USA, 84:6795-6799 (1987)) was used for all the control transfection experiments.

Percutaneous Arterial Gene Transfer in Vitro.

An interval of 10 days between the time of surgery and gene transfer was allowed for post-operative recovery of rabbits and development of endogenous collateral vessels. Beyond this time-point, studies performed up to 90 days post-operatively (Pu, et al., J. Invest. Surg., (In Press)) have demonstrated no significant collateral vessel augmentation. At 10 days post-operatively (day 0), after performing a baseline angiogram (see below), the internal lilac artery of the ischemic limb of 8 animals was transferred with phVEGF.sub.165 percutaneously using a 2.0 mm hydrogel-coated balloon catheter (Slider.TM. with HYDROPLUS,.RTM. Boston Scientific, Watertown, Mass.). The angioplasty balloon was prepared (ex vivo) by first advancing the deflated balloon through a 5 Fr. teflon sheath (Boston Scientific), applying 400 .mu.g of phVEGF.sub.165 to the 20 .mu.m-thick later of hydrogel on the external surface of the inflated balloon, and then retracting the inflated balloon back into the protective sheath. The Sheath and angioplasty catheter were then introduced via the right carotid artery, and advanced to the lower abdominal aorta using a 0.014 inch guidewire (Hi-Torque Floppy II, Advanced Cardiovascular Systems, Temecula, Calif.) under fluoroscopic guidance. The balloon catheter was then advanced out of the sheath into the internal lilac artery of the ischemic limb, inflated for 1 min at 6 atmospheres, deflated, and withdrawn (FIG. 1(a), open arrow). An identical protocol was employed to transfect the internal iliac artery of 9 control animals with the plasmid pGSVLacZ containing a nuclear targeted .beta.-galactosidase sequence. Heparin was not administered at the time of transfection or angiography.

Evaluation of Angiogenesis in the Ischemic Limb.

Development of collateral vessels in the ischemic limb was serially evaluated by calf blood pressure measurement and internal lilac arteriography immediately prior to transfection (day 0), and then in serial fashion at days 10 and 30 post-transfection. On each occasion, it was necessary to lightly anesthetize the animal with a mixture of Ketamine (10 mg/kg) and acepromazine (0.16 mg/kg) following premedication with xyazine (2.5 mg/kg). Following the final 30-day follow-up, the animal was sacrificed, and tissue sections were prepared from the hindlimb muscles in order to perform analysis of capillary density. These analyses are discussed in detail below.

Calf Blood Pressure Ratio.

Calf blood pressure was measured in both hindlimbs using a Doppler Flowmeter (Model 1050, Parks Medical Electronics, Aloha, Oreg.), immediately prior to transfection (day 0), as well as on days 10 and 30. On each occasion, the hindlimbs were shaved and cleaned; the pulse of the posterior tibial artery was identified using a Doppler probe; and the systolic pressure of both limbs was determined using standard techniques. Takeshita, et al., J. Clin. Invest., 93:662-670 (1994). The calf blood pressure ratio was defined for each rabbit as the ratio of systolic pressure of the ischemic limb to systolic pressure of the normal limb.

Selective Internal Iliac Arteriography.

Collateral artery development in this ischemic hindlimb model originates from the internal lilac artery. Accordingly, selective internal lilac arteriography was performed on day 0 (immediately prior to transfection), and again on days 10 and 30 post-transfection as previously described. Takeshita, et al., J. Clin. Invest., 93:662-670 (1994). A 3 Fr. end-hole infusion catheter (Tracker-18, Target Therapeutics, San Jose, Calif.) was introduced into the right common carotid artery through a small cutdown, and advanced to the internal lilac artery at the level of the interspace between the seventh lumbar and the first sacral vertebrae. Following intra-arterial injection of nitroglycerin (0.25 mg, SoloPak Laboratories, Franklin Park, Ill.), a total of 5 ml of contrast media (Isovue-370, Squibb Diagnostics, New Brunswick, N.J.) was then injected using an automated angiographic injector (Medrad, Pittsburgh, Pa.) programmed to reproducibly deliver a flow rate of 1 ml per sec. Serial images of the ischemic hindlimb were then recorded on 105-mm spot film at a rate of 1 film per sec for at least 10 sec. Following completion of arteriography, the catheter was removed and the wound was closed. All of the above-described procedures were completed without the use of heparin.

Morphometric angiographic analysis of collateral vessel development was performed as previously described. Takeshita, et al., J. Clin. Invest., 93:662-670 (1994). A composite of 5-mm.sup.2 grids was placed over the medical thigh area of the 4-sec angiogram. The total number of grid intersections in the medical thigh area, as well as the total number of intersections crossed by a contrast-opacified artery were counted individually by a single observer blinded to the treatment regimen. An angiographic score was calculated for each film as the ratio of grid intersections in the medial thigh.

Capillary Density and Capillary/Myocyte Ratio.

The effect of VEGF gene transfer upon anatomic evidence of collateral artery formation was further examined by measuring the number of capillaries in light microscopic sections taken from the ischemic hindlimbs. Takeshita, et al., J. Clin. Invest., 93:662-670 (1994). Tissue specimens were obtained as transverse sections from the ischemic limb muscles at the time of sacrifice (day 30 post-transfection). Muscle samples were embedded in O.C.T. compound, (Miles, Elkhart, Ind.) and snap-frozen in liquid nitrogen. Multiple frozen sections (5 .mu.m in thickness) were then cut from each specimen on a cryostat (Miles), so that the muscle fibers were oriented in a transverse fashion, and two sections then placed on glass slides. Tissue sections were stained for alkaline phosphate using an indoxyl-tetrazolium method to detect capillary endothelial cells (Ziada, et al., Cardiovasc. Res., 18:724-732 (1984)), and were then counterstained with eosin. Capillaries were counted under a 20x objective to determine the capillary density (mean number of capillaries per mm.sup.2). A total of 20 different fields was randomly selected, and the number of capillaries counted. To ensure that analysis of capillary density was not overestimated due to muscle atrophy, or underestimated due to interstitial edema, capillaries identified at necropsy were also evaluated as a function of myocytes in the

histologic section. The counting scheme used to compute the capillary/myocyte ratio was otherwise identical to that used to compute capillary density.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR), Southern Blot Analysis, and Sequencing of RT-PCR Product.

The presence of human VEGF mRNA was detecting using RT-PCR. Arterial samples were obtained at 5 days post-transfection, and total cellular RNA was isolated using TRI REAGENT (Molecular Research Center, Cincinnati, Ohio) according to the manufacturer's instructions. Extracted RNA was treated with DNase I (0.5 .mu.l, 10 U/.mu.l, RNase-free, Message Clean kit, GenHunter, Boston, Mass.) at 37.degree. C. for 30 min to eliminate DNA contamination. The yield of extracted RNA was determined spectrophotometrically by ultraviolet absorbance at 260 nm. To check that the RNA was not degraded and electrophoresed through a 1% non-denaturing miniagarose gel. 0.5 .mu.g of each RNA sample was used to make cDNA in a reaction volume of 20 .mu.l containing 0.5 mM of each deoxynucleotide triphosphate (Pharmacia, Piscataway, N.J.), 10 mM dithiothreitol, 10 units of RNasin (Promega, Madison, Wis.), 50 mM Tris-HCI (pH 8.3), 75 mM KCL, 3 mM MgCl.sub.2, 1 .mu.g random hexanucleotide primers (Promega), and 200 units of M-MLV reverse transcriptase (GIBCO BRL). For greater accuracy and reproducibility, master mixes for a number of reactions were made up and aliquoted to tubes containing RNA. Reactions were incubated at 42.degree. C. for 1 hr, then at 95.degree. C. for 5 min to terminate the reaction. Twenty .mu.l of diethyl pyrocarbonate (DEPC) water was then added and 5 .mu.l of the diluted reaction (1/8th) was used on the PCR analysis. The optimized reaction in a total volume of 20 .mu.l contained 0.2 mM of each deoxynucleotide triphosphate, 3 mM MgCl.sub.2, 2 .mu.l PCR II buffer (Perkin-Elmer, Norwalk, Conn.; final concentrations, 50 mM KCL, 10 mM Tris-HCL), 5 ng/.mu.l (13.77 pmoles) of each primer, and 0.5 units of AmpliTaq DNA polymerase (Perkin-Elmer). The PCR was performed on a 9600 PCR system (Perkin-Elmer) using microamp 0.2. ml thin-walled tubes. Amplification was for 40-45 cycles of 94.degree. C. for 20 sec, 55.degree. C. for 20 sec, and 72.degree. C. for 20 sec, ending with 5 min at 72.degree. C. To test for false positives, controls were included with no RNA and no reverse transcriptase. A pair of oligonucleotide primers (22 mers) was designed to amplify a 258 bp sequence from the mRNA of human VEGF. To ensure specificity and avoid amplification of endogenous rabbit VEGF, each primer was selected from a region which is not conserved among different species. Sequences of primers used were: 5'-GAGGGCAGAATCATCACGAAGT-3' (sense) SEQ. ID NO:1 ; 5'-TCCTATGTGCTGGCCTTGGTGA-3' (antisense) SEQ. ID NO:2. RT-PCR products were transferred from agarose gels to nylon membranes (Hybond, Amersham, Arlington Heights, Ill.). The probe was 5' end-labelled with T4 polynucleotide kinase and [.beta.-.sup.32 P]ATP (Sambrook, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, (1989)) and hybridized to the nylon filters using Rapid Hybridization buffer (Amersham) according to manufacturer's instructions. To visualize hybridized bands, filters were exposed to X-ray film (Kodak Xar-5).

To confirm the identity of VEGF PCR products. DNA bands were excised from agarose gels, purified using Geneclean (BIO 101, La Jolla, Calif.), and sequenced directly (i.e. without subcloning) using dsDNA Cycle Sequencing System (GIBCO BRL) following the directions of manufacturer. The two VEGF primers used for PCR were 5' end-labeled with [.beta.-.sup.32 P]ATP and T.sub.4 polynucleotide kinase and used as sequencing primers to determine the sequence of both strands of the PCR product.

.beta.-Galactosidase Staining of Transfected lilac Arteries.

To evaluate the efficiency of in vivo arterial gene transfer, .beta.-galactosidase activity was determined

by incubation of arterial segments with 5-bromo-4-chloro-3-indolyl .beta.-D-galactosidase chromogen (X-Gall), Sigma) as previously described. Riessen, et al., Hum. Gene Ther., 4:749-758 (1993). Following staining with X-Gal solution, tissues were paraffin-embedded, sectioned, and counterstained with nuclear fast red. Nuclear localized .beta.-galactosidase expression of the plasmid pGSVLacZ cannot result from endogenous .beta.-galactosidase activity; accordingly, histochemical identification of .beta.-galactosidase within the cell nucleus was interpreted as evidence for successful gene transfer and gene expression. Cytoplasmic or other staining was considered non-specific for the purpose of the present study.

Statistics.

Results were expressed as means.+-.standard deviation (SD). Statistical significance was evaluated using unpaired Student's t test for more than two means. A value of $p<0.05$ was interpreted to denote statistical significance.

Results

ELISA Assay for VEGF. To test the expression of the plasmid phVEGF.sub.165 in vascular cells, culture supernatant of VEGF-transfected SMCs (1.48.times.10.sup.6 cells/10 cm plate) was sampled at 3 days post-transfection, and analyzed by ELSA for VEGF protein. The media of VEGF-transfected SMCs contained an average of 1.5 .mu.g of VEGF protein (n=3). In contrast, culture media of .beta.-galactosidase-transfected SMCs (n=3) or non-transfected SMCs (n=3) did not contain detectable levels of VEGF protein.

RT-PCR, Southern Blot Analysis, and Sequencing of RT-PCR Product.

To confirm expression of human VEGF gene in transfected rabbit lilac arteries in vivo, we analyzed transfected arteries for the presence of human VEGF mRNA by RT-PCR. As indicated above, to ensure the specificity of RT-PCR for human VEGF mRNA resulting from successful transfection (versus endogenous rabbit VEGF mRNA), primers employed were selected from a region which is not conserved among different species. Arteries were harvested at 5 days post-transfection. The presence of human VEGF mRNA was readily detected in rabbit SMC culture (n=3) and rabbit lilac arteries (n=3) transfected with phVEGF.sub.165. Rabbit lilac arteries transfected with pGSVLacZ (n=3) were negative for human VEGF mRNA (FIG. 2(a)). Southern blot analysis was used to further confirm that the 158 bp bands obtained by RT-PCR did in fact correspond to the region between the two primers (FIG. 2(b)). Direct sequencing of the RT-PCR product document that this band represented the human VEGF sequence (FIG. 2(c)).

Angiographic Assessment.

The development of collateral vessels in the 5 rabbits transfected with phVEGF.sub.165 and 6 rabbits transfected with pGSVLacZ was evaluated by selective internal lilac angiography. FIG. 3 illustrates representative internal lilac angiogram recorded from both control and VEGF-transfected animals. In control animals, collateral artery development in the medial thigh typically appeared unchanged or progressed only slightly in serial angiogram recorded at days 0, 10, and 30 (FIGS. 3(a-c)). In contrast, in the VEGF-transfected group, marked progression of collateral artery was observed between days 10 and 30 (FIGS. 3, (d-f)). Morphometric analysis of collateral vessel development in the media thigh was performed by calculating the angiographic score as described above. At baseline (day 0), there was no significant difference in angiographic score between the VEGF-transfected and control groups (day 0:0.17.+-.0.02 vs 0.20.+-.0.06, p=ns). By day 30, however, the angiographic score in VEGF-

transfected group was significantly higher that in control group (0.47.+-.0.09 vs 0.34.+-.0.10, p<0.05) (FIG. 4(a)).

Calf Blood Pressure Ratio (FIG. 4(b)).

Reduction of the hemodynamic deficit in the ischemic limb following VEGF-transfection was confirmed by measurement of calf blood pressure ratio (ischemic/normal limb). The calf blood pressure ratio was virtually identical in both groups prior to transfection (0.23.+-.0.12 in VEGF-transfected animals, p=ns). By day 10 post-transfection, the blood pressure ration for VEGF-transfected rabbits was significantly higher than for the control rabbits (0.60.+-.0.12 vs 0.32.+-.0.14, p<0.01). At day 30, the blood pressure ratio for the VEGF-transfected group continued to exceed that of controls (0.70.+-.0.08 vs 0.50.+-.0.18, p<0.05).

Capillary Density and Capillary/Myocyte Ratio (FIGS. 4(c), 5).

A favorable effect of VEGF-transfection upon revascularization was also apparent at the capillary level. The medial thigh muscles of the ischemic limbs were histologically examined at day 30 post-transfection. Analysis of capillary density disclosed a value of 233.0.+-.60.9/mm.sup.2 in VEGF-transfected group versus 168.7.+-.31.5/mm.sup.2 in the control group (p<0.05). Analysis of capillary/myocyte ratio disclosed a value of 0.67.+-.0.15 in the VEGF-transfected group versus 0.48.+-.0.10 in the control group (p<0.05).

.beta.-Galactosidase Staining of Transfected Iliac Arteries.

To evaluate the efficiency of in vivo arterial gene transfer, transfected lilac arteries were harvested at 5 days post-transfection, and were used for .beta.-galactosidase histochemical analysis. In arteries transfected with nuclear targeted .beta.-galactosidase, evidence of successful transfection, indicated by dark blue nuclear staining, was observed in only<0.5% of total arterial cells. Arteries transfected with phVEGF.sub.165 were negative for nuclear staining.

EXAMPLE 3

Comparison of Double-Balloon Catheter Technique and Hydrogel-Coated Balloon Catheter Technique

Methods

Recombinant Adenoviral Vectors

Replication-defective recombinant adenoviral vectors, based on human adenovirus 5 serotype, were produced as previously described. Quantin, et al., Proc. Nat. Acad. Sci. USA, 89:2581-2584 (1992); Stratford-Perricaudet, et al., J. Clin. Invest., 90:626-630 (1992); and Rosenfeld, et al., Cell, 68:143-155 (1992). Ad-RSV.beta.gal contains the Escherichia coil lac Z gene and the SV40 early region nuclear localization sequence (nls). The nlslacZ gene encodes a nuclear-targeted .beta.-galactosidase under the control of the Rous sarcoma virus promoter. Ad-RSVmDys, used as a negative control, contains a human "minidystrophin" cDNA under the control of the same promoter. Ragot, et al., Nature, 361:647-650 (1993).

In Vivo Percutaneous Gene Transfer Procedures

All animal procedures were approved by the Institutional Animal Care and Use Committees of Faculte Bichat and St. Elizabeth's Hospital. Gene transfer was performed in the external lilac artery of 29 New Zealand white rabbits under general anesthesia and sterile conditions. Anesthesia was induced with intramuscular acepromazine and maintained with intravenous pentobarbital. Adenoviral stocks were used within 30 minutes of thawing.

1. Double-balloon catheter technique.

In 15 animals, Ad-RSV.beta.gal (2.10.sup.9 to 2.10.sup.10 plaque forming unites {pfu} in 2 ml PBS) was transferred to the right lilac artery, either normal (n=9) or previously denuded (n=6), using a 4 French double-balloon catheter (Mansfield Medical, Boston Scientific Corp., Watertown, Mass.) as previously described. Nabel, et al., Science, 244:1342-1344 (1989). The catheter was positioned in a segment of the artery which lacked angiographically visible side branches. The viral solution was maintained in contact with the arterial wall for 30 min. The left lilac artery of the same 15 animals was used as a control: in 7 animals no catheter was inserted, in 6 animals the endothelium was removed using balloon abrasion, and, in the 2 other animals, a double-balloon catheter was used in infuse Ad-RSVmDys (2.10.sup.9 pfu in 2 ml PBS).

2. Hydrogel-Coated Balloon Catheter Technique.

In 14 animals, a hydrogel-coated balloon catheter was used (Slider.TM. with Hydroplus.RTM., Mansfield Medical, Boston Scientific Corp., Watertown, Mass.). The balloon diameter (either 2.5 or 3.0 mm), was chosen to approximate a 1.0 balloon/artery ratio based on caliper measurement of magnified angiographic frames. Ad-RSV.beta.gal (1-2.10.sup.10 pfu in 100 .mu.l PBS) was applied to the polymer-coated balloon using a pipette as described above. The catheter was introduced into the right femoral artery through a protective sheath, the balloon was inflated at 1 atm, and the assembly was then advanced over a 0.014" guide wire to the external lilac artery where, after balloon deflation, the catheter alone was advanced 2 cm further and the balloon inflated for 30 minutes at 6 atm (ensuring nominal size of the inflated balloon). The contralateral lilac artery was in each case used as a control: in 9 animals no catheter or virus was introduced, in 2 the endothelium was removed, while in 3 a hydrogel-coated balloon catheter was used to transfer Ad-RSVmDys.

Detection of lacZ Expression in the Arterial Wall.

Three to seven days after transfection, the animals were sacrificed by pentobarbital overdose. To assess nlslacZ gene expression, the arteries were harvested and stained with X-Gal reagent (Sigma) for 6 hours, at 32.degree. C., as previously described. Sanes, et al., EMBO J., 5:3133-3142 (1986). Samples were then either mounted in OCT compound (Miles Laboratories Inc., Ill.) for cryosectioning or embedded in paraffin, cut into 6-.mu.m sections, and counterstained with hematoxylin and eosin or elastic trichrome. Expression of nlslacZ gene was considered positive only when dark blue staining of the nucleus was observed. To determine which cell types within the arterial wall expressed the transgene, immunohistochemical staining of X-Gal-stained arterial sections was performed, using a mouse monoclonal anti-.alpha.-actin primary antibody specific for vascular smooth muscle (HHF-35, Enzo Diagnostics, Farmingdale, N.Y.), and then a polyclonal peroxidase-labeled anti-mouse immunoglobulin G secondary antibody (Signet Laboratories, Dedham, Mass.).

Morphometric Analysis of nlslacZ Gene Expression in the Media.

For each transfected iliac artery, at least 2 samples were taken from the target-zone, and from each sample, at least 3 sections were examined by light microscopy after X-gal staining. Due to the

heterogeneity of .beta.-galactosidase activity on gross examination, the percentage of transfected medial cells per artery section was determined in regions showing high .beta.-galactosidase activity by counting stained versus total nuclei. The total numbers of studied medial cells were 14.10.sup.3 (n=50 sections) in the double-balloon catheter and the hydrogel-coated balloon catheter groups respectively.

Detection of Remote.sup.nlslacZ Gene Transfer and Expression.

Tissue samples from liver, brain, testes, heart, lungs, kidneys, contralateral limb skeletal muscle, and arterial segments adjacent to the treated arterial site were harvested immediately after sacrifice. For each specimen, nlslacZ gene presence and expression were assessed by polymerase chain reaction (PCR) and histochemistry (X-gal staining) respectively.

For PCR, genomic DNA was extracted from tissues by standard techniques. DNA amplification was carried out using oligodeoxynucleotide primers designed to selectively amplify Ad-RSV.beta.gal DNA over endogenous .beta.-galactosidase gene by placing one primer in the adenovirus sequence coding for protein 9 and the other primer in the lacZ sequence (5'-AGCCCGTCAGTATCGGCGGAATTC-3' (SEQ ID NO:3) and 5'-CAGCTCCTCGGTCACATCCAG-3' (SEQ ID NO:4) respectively, Genset, Paris, France). The reactions were performed in a DNA thermocycler (GeneAmp PCR System 9600, Perkin Elmer Cetus, Norwalk, Conn.) following 2 different protocols: a hold at 95.degree. C. for 3 min, 35 or 45 cycles of 95.degree. C. for 30 s, 65.degree. C. for 40 s, and 72.degree. C. for 1 min, then a final extension at 72.degree. C. for 5 min. When PCR was performed on plasmid DNA containing the nlslacZ gene used for the preparation of the adenoviral vector, or on positive liver samples obtained by deliberate systemic injection of Ad-RSV.beta.gal, the amplification reaction produced a 700 bp DNA fragment. To determine sensitivity of these procedures, DNA was extracted from liver of uninfected rabbits, aliquoted into several tubes, and spiked with dilutions of the plasmid containing the nlslacZ gene and used as a positive control. Following the amplification protocols described above, it was determined that the 35- or 45-cycle PCR could detect one copy of the nlslacZ gene in 3.102 and 3.104 cells respectively. DNA extractions and amplifications were performed simultaneously and in duplicate for studied tissues and positive controls.

Each tissue sample was also processed for histochemical analysis following the same protocol described for the arteries. For each specimen, at least 3 different segments were obtained, embedded in paraffin, and cut into at least 5 sections. Sections were counterstained with hematoxylin and eosin, and examined by light microscopy for the presence of deep blue nuclei indicative of .beta.-galactosidase expression. The number of positive cells as well as the total number of cells were counted. The total number of cells examined per sample ranged from 25.10.sup.3 to 115.10.sup.3.

Statistics

Results are expressed as mean.+-.standard deviation (SD). Comparisons between groups were performed using Student's t test for unpaired observations. A value of p<0.05 was accepted to denote statistical significance.

Results

Histological and Histochemical Analyses of Transfected Arteries Following Double-Balloon Catheter Delivery

Gross examination of all the arteries (n=15) following X-gal staining showed punctiform, heterogeneous, blue staining on the luminal aspect of the arteries, always limited to the area between

the two balloons. For the 9 normal arteries, microscopic examination disclosed dark blue nuclear staining, confined entirely to the endothelium. In contrast, when endothelial abrasion preceded transfection (n=6), X-gal staining imparted a mottled appearance to the luminal aspect of the artery. In these cases, microscopic examination showed that the endothelium had been removed and that the site of X-gal staining was subjacent to the intact internal elastic lamina, involving sparse medial cells. The identity of the transfected medial cells as smooth muscle cells was confirmed by immunohistochemical staining with monoclonal anti-.alpha.-actin antibody. Control arteries showed no nuclear blue staining.

Histological and Histochemical Analysis of Transfected Arteries Following Hydrogel-Coated Balloon Catheter Delivery

Gross examination of all the arteries after X-gal staining (n=14) showed dark blue, heterogeneous staining of the transfected site with a sharp boundary between the transfected segment and the bordering proximal and distal segments. Microscopic examination showed no residual intact endothelium; the continuity of the internal elastic lamina, in contrast, appeared preserved without apparent disruption. In the areas showing evidence of .beta.-galactosidase activity on gross examination, light microscopic examination revealed nearly continuous layers of cells with dark blue nuclear staining, generally limited to the superficial layers of the media; occasionally, sparsely distributed cells from deeper layers of the media expressed the transgene as well. Staining with monoclonal anti-.alpha.-actin antibody confirmed that transfected cells were vascular smooth muscle cells. No evidence of nuclear .beta.-galactosidase activity was seen in control arteries.

Morphometric Analysis of nlslacZ Gene Expression in the Media.

The percentage of transduced cells per artery section in regions showing high .beta.-galactosidase activity was significantly higher in the hydrogel-coated balloon catheter group than in the double-balloon catheter group (6.1.+-.2.3% vs. 0.4.+-.0.6%, p<0.0001).

Detection of Remote lacZ Gene Transfer and Expression in Other Organs

In all animals of both groups, gross and microscopic examination of X-gal stained tissue samples from liver, brain, testes, heart, lungs, kidneys, contralateral limb skeletal muscle, and arterial segments adjacent to the treated arterial site failed to show expression of nuclear-targeted .beta.-galactosidase, except in the liver of one rabbit in the double-balloon catheter group which disclosed a limited area of nuclear and peri-nuclear blue staining. In this area, less than 1/2.10.sup.3 cells expressed .beta.-galactosidase. In a few macrophages limited to samples removed from the lungs and testes of one hydrogel-coated balloon catheter treated rabbit, blue staining of the cytoplasm without nuclear staining was observed; the exclusively cytoplasmic location of .beta.-galactosidase activity in these cases, however, suggested that staining resulted from endogenous .beta.-galactosidase.

All of the above tissue samples were then screened by PCR. When the PCR was run for 35 cycles, the presence of DNA sequence specific for Ad-RSV.beta.gal was non-detectable, including in tissue samples from those animals with the highest percentage of transfected lilac arterial cells. Using an optimized protocol of 45 cycles, however, PCR was positive in the single liver that was observed to express .beta.-galactosidase, but in none of the other tissues.

This invention has been described in detail including the preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements thereon without departing from the spirit and scope of the invention as set forth in the claims.

HARVARD UNIVERSITY Gazette

The following articles appeared in the May 14, 1998, issue. Brief items have been omitted.

College Admission Yield Is Nearly 80%

Women's Studies in Religion Brings New Voices, Perspectives

Bone Drug Lowers Risk of Heart Disease

Virtual Press Room Open for Harvard Conference on Internet & Society

Notes

Labor Economist Myra Strober to Deliver Feminist Economics Lecture at Radcliffe Institute

Police Blotter

A Life of Service

NewsMakers

Peiser Appointed as Professor at Graduate School of Design

Study Finds that Governmental Procedure To Reduce Litigation Actually Leads to More Lawsuits

Knowles Elected Trustee Of Howard Hughes Medical Institute

Faculty To Meet with South Africa's Desmond Tutu, Truth Commission

'Radrugby': Bruised, Battered, Unbowed

Fragments of a Forgotten Past

FAS Administrative, Professional Prizes Honor Staff

New Harvard Features Service Goes Online

EXHIBIT C-6

Seven Students Win Paine Fellowships

Dental Center's Faculty Practice What They Teach

3/26/99 3:20 PM

New Arteries Grown In Diseased Hearts

By William J. Cromie

Gazette Staff

Almost anything Hugh Curtis did gave him a pain in the heart. Even when lying in bed, he felt the stabbing chest pains of angina, a hurtful signal that his heart was not getting enough oxygen.

Curtis underwent a quadruple bypass in 1986, then a single bypass late last year. Surgeons removed veins from his legs and grafted them onto his heart to bypass his blocked coronary arteries. But that didn't solve his problem.

He also received a series of angioplasties, wherein tiny balloons were threaded into his heart's arteries, then inflated. This process pushed the blockages aside, opening his arteries. Five pieces of metal mesh were installed to keep them open, but his coronary arteries closed in other places.

"I couldn't walk very far, couldn't even make my bed," says the 55-year-old resident of Danvers, Mass. "Climbing stairs was out, so was any thought of going on vacation."

Late last year, he was asked by researchers at Beth Israel Deaconess Medical Center in Boston if he wanted to volunteer for an experimental procedure at the Harvard-affiliated hospital. The procedure involved doctors injecting proteins called growth factors into his heart to stimulate growth of new blood around those clogged with plaque.

"I didn't hesitate to give them the go-ahead," Curtis recalls.

The cardiologists threaded a thin hollow tube from his groin into his heart. Through the tube they injected what is called basic fibroblast growth factor, or bFGF.

Four months after the treatment, Curtis is back working full time at a desk job in a printing company. "I no longer take 3-to-6 nitroglycerin tablets a day, and I'm painting the hallway in my house," he says cheerily. "I may never go back to playing racquetball, but I'm leading a normal life, and that's all I'm looking for."

"All his symptoms are gone," says Michael Simons, associate professor of medicine at Harvard Medical School. "He is one of 18 patients who participated in a trial of bFGF. All are now largely without

symptoms such as chest pain, shortness of breath, and fatigue."

Bypassing Bypass Surgery

Eighteen other patients who received heart-artery bypasses got bFGF at the same time. Frank Sellke, an associate professor of surgery at Harvard Medical School, implanted capsules that slowly release the drug at sites where blocked vessels were too small or too diffusely diseased to bypass.

"These patients have undergone treadmill stress tests," Simons comments. "They also have been examined with a new type of magnetic resonance imaging (MRI) that measures blood flow and detects new vessel development. It is too early to scream and shout with success, but we are pleased with the results obtained so far."

"I had an MRI a couple of weeks ago, and it showed new arteries growing and bypassing some blockage," says Curtis. "I'm getting 70 percent blood flow to an area of the heart that was down to 30 percent flow. And there's reason to think things will improve more with time."

John Modugno, 48, received bFGF in February, and his MRI tests also show evidence of new arterial growth. "I feel much better," he says, "although I'm still on drugs and get a little angina at the end of the day."

Tests of bFGF and other growth factors now under way at various research centers raise hopes that newly grown blood vessels will replace arteries choked off by atherosclerosis, thus heading off thousands, maybe millions, of heart failures and heart attacks.

If these tests continue to be successful in humans, they could lead to heart drugs that will be cheaper, safer, and a lot easier on patients than bypass surgery and angioplasty. About a million people undergo such procedures in the United States each year, but they don't always work. As in Hugh Curtis's case, some vessels are too small or located where they can't be bypassed with sections of vein. After arteries have been opened by an inflated balloon or other types of angioplasty, about one-third of them close again, some in a matter of months.

"We once thought people in which neither procedure worked accounted for only a small subgroup of patients," Simons says. "But now we're getting phone calls almost every day, so we must conclude that there are more people with this problem than we imagined."

The revolutionary potential of growth factors, of course, goes far beyond such people. Simons sees it as "having the potential to replace or reduce the use of bypass surgery." The American Heart Association estimates that 500,000 bypasses are performed each year at an average cost of $45,000 per treatment.

Severely blocked coronary arteries cause more than 3 million heart failures a year, and 7 million more people suffer the chest pains of angina. "Growth-factor treatments might be expanded to many, if not all, of these patients," Simons declares.

The Side-Effects Question

Researchers at Beth Israel Deaconess Medical Center initiated such treatments in 1996. Today, seven

teams worldwide work on growing new blood vessels with bFGF and another protein known as vascular endothelial growth factor, or VEGF (see April 23 *Gazette,* page 1).

In a trial conducted at several medical centers, VEGF was given to 17 people whose blocked coronary arteries lay out of reach of angioplasty. Fifteen of the 17 patients showed various levels of improvement.

Jeffrey Isner, a cardiologist at St. Elizabeth's Medical Center in Boston, has used VEGF to grow new vessels around blockages in the leg veins of diabetics. He has treated 30 diabetic patients, as well as five other patients with heart disease.

"Preliminary results look good in both types of disease," Isner says. "This is a very encouraging and exciting area of treatment."

The great promise of bypassing blood-vessel blockages won't become a reality, however, if the growth factors cause severe side effects.

Both bFGF and VEGF lower blood pressure. "That fact limits the amount you can give a person," Simons notes. "But that's something we can work around."

More serious is the possibility of damage to sight caused by overgrowth of blood vessels in the eye. "We have been looking carefully for this, but have not seen any as yet with bFGF," Simons comments. Also, no new blood vessels were seen growing in the eyes of patients treated with VEGF, another encouraging sign.

The most worrisome possibility concerns growth of blood vessels that might nourish small, hidden cancer tumors. Judah Folkman, another Harvard researcher, found that such tumors remain benign unless new blood vessels carry nutrients to them. Once connected to a steady blood supply, tumors grow and spread.

Folkman and Michael O'Reilly developed two exciting new cancer drugs, endostatin and angiostatin, which block rather than promote development of blood vessels.

"We hope that tumor growth can be avoided because we give the growth factor for a very short time and in small amounts," Simons notes. "It's not like we're adding a foreign substance to the body; everyone has such small amounts of bFGF circulating naturally in their bloodstream."

The side-effects issue will be addressed in tests involving larger numbers of patients. Plans call for testing both growth factors on 400 to 500 people at a combination of medical centers throughout the country. Simons expects to start expanded trials of bFGF this summer in a collaboration with Emory University in Atlanta.

A question still to be answered is exactly how new blood vessels form. The bare-bones explanation has bFGF binding to the surface of, then stimulating growth of endothelial cells, those that line the inside of capillaries, the smallest vessels. These cells leave the vessels, migrate to the tip of the capillaries, and form a tube that extends their reach.

Simons's team took startling photos of new vessels growing around blocked arteries in animals. They show small extensions sprouting like twigs on a tree limb, moving around the barricade and reconnecting on the other side.

"It's amazing to see," Simons says. "If we can continue to do the same thing in humans, without deleterious side effects, we have a chance to benefit millions of people."

END

College Admission Yield Is Nearly 80%

Highest in 25 years

Nearly 80 percent of students admitted to the Class of 2002 have chosen to enroll, the highest yield since the early 1970s, according to the Undergraduate Admissions Office. This yield is the best in more than 25 years.

Yield, the percentage of admitted candidates who decide to accept an offer of admission, is considered a measure of a school's competitiveness. Harvard's yield is again, by a wide margin, the highest of the nation's selective colleges. When the final figures are available, the yield could go even higher -- it is already well above last year's yield of 76.3 percent.

The 2,073 students admitted to the Class of 2002 were selected from a pool of 16,819 applicants. For the seventh time in eight years, applications for admission to Harvard and Radcliffe have risen. Last year, 16,597 students applied for the 1,650 places in the entering class.

The substantial rise in the yield means that the Class of 2002 is now full, and it will probably be impossible to admit anyone from the waiting list. In more typical years, the College has been able to admit between 50 and 100 from the waiting list.

"We are extremely pleased that the College has again attracted so many extraordinarily talented students this year," said William R. Fitzsimmons '67, Dean of Admissions and Financial Aid. "With many leading American and international universities recently announcing changes in their financial aid programs designed to compete more aggressively for top students, the leadership of Dean of the Faculty of Arts and Sciences Jeremy Knowles and President Neil Rudenstine allowed Harvard to extend its best welcome to prospective members of the Class of 2002."

The Dean and President reemphasized their unwavering commitment to a strong need-based financial aid program and to the policy of admitting the best students without regard to their financial circumstances. With nearly 70 percent of all undergraduates on financial aid, and with scholarship grants of $45 million, Harvard has always been a leader in financial aid.

Dean Knowles stated in February, "We shall set no limit on the financial resources necessary to make Harvard College fully accessible to all students of promise. . . Students who are admitted to next fall's entering class will receive competitively supportive offers, and financial aid will be tailored flexibly and individually."

James S. Miller, director of financial aid, and his staff were available weekdays from 8 a.m. to 8 p.m. and several Saturdays for the month of April, and talked with an unprecedented number of students and parents about their financial aid awards. "Jim and his staff worked extremely hard to make it possible for

EXHIBIT C-7

Early reports

Clinical evidence of angiogenesis after arterial gene transfer of phVEGF$_{165}$ in patient with ischaemic limb

Jeffrey M Isner, Ann Pieczek, Robert Schainfeld, Richard Blair, Laura Haley, Takayuki Asahara, Kenneth Rosenfield, Syed Razvi, Kenneth Walsh, James F Symes

Summary

Background Preclinical findings suggest that intra-arterial gene transfer of a plasmid which encodes for vascular endothelial growth factor (VEGF) can improve blood supply to the ischaemic limb. We have used the method in a patient.

Methods Our patient was the eighth in a dose-ranging series. She was aged 71 with an ischaemic right leg. We administered 2000 μg human plasmid phVEGF$_{165}$ that was applied to the hydrogel polymer coating of an angioplasty balloon. By inflating the balloon, plasmid DNA was transferred to the distal popliteal artery.

Findings Digital subtraction angiography 4 weeks after gene therapy showed an increase in collateral vessels at the knee, mid-tibial, and ankle levels, which persisted at a 12-week view. Intra-arterial doppler-flow studies showed increased resting and maximum flows (by 82% and 72%, respectively). Three spider angiomas developed on the right foot/ankle about a week after gene transfer; one lesion was excised and revealed proliferative endothelium, the other two regressed. The patient developed oedema in her right leg, which was treated successfully.

Interpretation Administration of endothelial cell mitogens promotes angiogenesis in patients with limb ischaemia.

Lancet 1996; **348**: 370–74

Introduction

Among the growth factors that promote angiogenesis, vascular endothelial growth factor (VEGF),[1] also known as vascular permeability factor,[2] and vasculotropin,[4] is specifically mitogenic for endothelial cells. The first exon of the VEGF gene includes a secretory signal sequence that permits the protein to be secreted naturally from intact cells.[5] We have shown[6,7] that arterial gene transfer of naked DNA encoding for secreted protein yielded physiological levels of protein despite low transfection efficiency. Site-specific gene transfer of plasmid DNA encoding the 165-aminoacid isoform of human VEGF (phVEGF$_{165}$) applied to the hydrogel polymer coating of an angioplasty balloon,[8] and delivered percutaneously to the iliac artery of rabbits in which the femoral artery had been excised to cause unilateral hindlimb ischaemia led to

Departments of Medicine, Biomedical Research, Radiology, and Surgery, St Elizabeth's Medical Center, Tufts University School of Medicine, Boston, Massachusetts, USA (Jeffrey M Isner MD, Ann Pieczek RN, Robert Schainfeld DO, Richard Blair MD, Laura Haley BS, Takayuki Asahara MD, Kenneth Rosenfield MD, Syed Razvi MD, Kenneth Walsh PhD, James F Symes MD)

Correspondence to: Dr Jeffrey M Isner, St Elizabeth's Medical Center, Boston, MA 02135, USA

development of collateral vessels and increased capillary density, improved calf blood-pressure ratio (ischaemic/normal limb) and increased resting and maximum vasodilator-induced blood flow.[7,10] We now use this strategy in the ischaemic limb of a patient.

Patient and methods

Patient

A 70-year-old non-diabetic woman was referred for gangrene of the right great toe. About a year earlier, the patient had cramping right-foot pain; several corns were removed, she was given intramuscular cortisone, prescribed ibuprofen, and fitted with shoe inserts. Symptoms worsened and the patient received oxycodone, hydrocodone, and a fentanyl patch. The great toe lesion progressed to gangrene, and the second and third toes became compromised. She had no palpable pedal pulses of the right limb. Ankle-brachial index of the ischaemic limb was 0·26. Arteriography revealed a 40% stenosis of the proximal popliteal artery, and occlusion of the peroneal, anterior tibial, and posterior tibial arteries midway to the foot. Surgical exploration of the distal right limb failed to identify a suitable site for a bypass.

The patient was suitable for arterial gene therapy according to a protocol[11] approved by the Human Institutional Review Board and Institutional Biosafety Committee of our centre, as well as the Recombinant DNA Advisory Committee of the National Institutes of Health and the US Food and Drug Administration.

Plasmid DNA

phVEGF$_{165}$ consists of a eucaryotic PUC 118 expression vector into which cDNA encoding the 165-aminoacid isoform of VEGF has been inserted.[1] A 763 basepair cytomegalovirus promoter/enhancer is used to drive VEGF expression. The PUC 118 vector includes an SV40 polyadenylation sequence, an *Escherichia coli* origin of replication, and the β-lactamase gene for ampicillin resistance. The plasmid was prepared in the Human Gene Therapy Laboratory at our centre from cultures of phVEGF$_{165}$-transformed *E coli*, purified with a Qiagen-tip 2500 column, precipitated with isopropanol, washed with 70% ethanol, and dried on a Speed Vac. The purified plasmid was reconstituted in sterile saline, stored in vials, and pooled for quality control analyses (absorbance at wavelengths of 260 and 280 nm to document ratio between 1·75 and 1·85; limulus amoebocyte lysate gel-clot assay [BioWhittaker] to establish bacterial endotoxin levels below 5 endotoxin units per kg bodyweight; microbial cultures; southern blot for level of contaminating genomic *E coli* DNA; and ethidium bromide staining after agarose-gel electrophoresis to confirm that over 90% of the nucleic acid was in the closed, circular supercoiled form). To confirm the identity of the prepared plasmid, the VEGF-coding region from each pooled batch was resequenced (Applied Biosystem 373A).

Percutaneous arterial gene transfer

Arterial gene transfer was done with a hydrogel-coated balloon-angioplasty-catheter (Boston Scientific).[8] A sterile pipette was used to apply 2000 μg plasmid DNA at 10·3 μg/μL in 194·2 μL

EXHIBIT D

DISCLOSURES

Growth factors can be utilized to induce the growth
of "hard tissue" or bone and "soft tissues" like ectoder-
mal and mesodermal tissues. As used herein, the term
growth factor encompasses compositions and living
organisms which promote the growth of hard tissue,
such as bone, or soft tissue in the body of a patient. The
compositions include organic and inorganic matter. The
compositions can be genetically produced or manipu-
lated. The living organisms can be bacteria, viruses, or
any other living organism which promote tissue
growth. By way of example and not limitation, growth
factors can include platelet-derived growth factor
(PDGF), epidermal growth factor (EGF), fibroblast
growth factor (acidic/basic)(FGF a,b), interleukins
(IL's), tumor necrosis factor (TNF), transforming
growth factor (TGF-B), colony-stimulating factor
(CSF), osteopontin (Eta-1 (OPN), platelet-derived
growth factor (PDGF), interferon (INF), bone mor-
phogenic protein 1 (BMP-1), and insulin growth factor
(IGF). Recombinant and non-recombinant growth fac-
tors can be utilized as desired. Bacteria or viruses can,
when appropriate, be utilized as growth factors. For
example, there is a bacterial hydrophilic polypeptide
that self-assembles into a nanometer internal diameter
pore to build a selective lipid body. Various enzymes
can be utilized for the synthesis of peptides which con-
tain amino acids that control three-dimensional protein
structure and growth. Growth factors can be applied in
gels or other carriers which regulate the rate of release
of the growth factors and help maintain the growth
factors, and the carrier, at a desired location in the
body. Time release capsules, granules, or other carriers
containing growth factor can be activated by tissue pH,
by enzymes, by ultrasound, by electricity, by heat, by
selected in vivo chemicals or by any other selected
means to release the growth factor. The carrier can be
resorbable or non-resorbable. Or, the growth factor
itself can be activated by similar means. Either the car-
rier or the growth factor can mimic extracellular fluid
to control cell growth, migration, and function. The
growth factor can be administered orally, systemically,
in a carrier, by hypodermic needle, through the respira-
tory tract, or by any other desired method. The growth
factor can also be administered into a capsule or other
man-made composition or structure placed in the body.
While administration of the growth factor is presently
usually localized in the patient's body, circumstances
may arise where it is advantageous to distribute a
growth factor throughout the patient's body in uniform
or non-uniform concentrations. An advantage to
growth factors is that they can often, especially when in
capsule form or in some other containment system, be
inserted to a desired site in the body by simply making
a small incision and inserting the growth factor. The
making of such a small incision comprises minor sur-
gery which can often be accomplished on an out-patient
basis. The growth factors can be multifactorial and
nonspecific.

In another embodiment of the invention, genetically
produced living material is used to form an implant in
the bone of a patient. The DNA structure of a patient is
analyzed from a sample of blood or other material ex-
tracted from a patient and a biocompatible tooth bud
122 (FIG. 3) is produced. The bud 122 is placed in an
opening 123 in the alveolar bone and packing material is
placed around or on top of the bud 122. The size of
opening 123 can vary as desired. The packing around
bud 122 can comprise HAC 124, hydroxyapatite, blood,
growth factors, or any other desirable packing material.
The bud 122 grows into a full grown tooth in the same
manner that tooth buds which are in the jaws of chil-
dren beneath baby teeth grow into full sized teeth. In-
stead of bud 122, a quantity of genetically produced
living material which causes bud 122 to form in the
alveolar bone can be placed at a desired position in the
alveolar bone such that bud 122 forms and grows into a
full sized tooth. Instead of forming an opening 123, a
needle or other means can be used to simply inject the
genetically produced living material into a selected
location in the alveolar bone. As would be appreciated
by those skilled in the art, genetically produced materi-
als can be inserted in the body to cause the body to
grow, reproduce, and replace leg bone, facial bone, and
any other desired soft and hard tissue in the body.

EVIDENCE APPENDIX

ITEM NO. 2

Declaration of Dr. Wayne H. Finley cited by Appellant as an
Exhibit in the Amendment filed February 15, 2001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: James P. Elia)
)
SERIAL NO.: 09/064,000) EXAMINER: Nicholas D. Lucchesi
)
FILED: April 21, 1998)
)
FOR: METHOD AND APPARATUS) GROUP ART UNIT: 3732
FOR INSTALLATION OF)
DENTAL IMPLANT)

DECLARATION OF WAYNE H. FINLEY, M.D.

I Wayne H. Finley declare as follows:

1. I reside at 3412 Brookwood Road, Mountain Brook, Alabama 35223.

2. My Curriculum Vitae is attached hereto as Exhibit A.

3. I have read and understood the disclosures at column 14, lines 4-61 and column 21, lines 1-26 of United States Patent Number 5,397,235 (hereinafter " '235 patent") entitled "Method for Installation of Dental Implant," and granted to James P. Elia on March 14, 1995. A copy of such disclosures is attached hereto as Exhibit D. I understand that the same disclosures are contained in above patent application Serial No. 09/064,000.

4. I note that the disclosures mentioned in above Paragraph 3 relate to a method for forming a bud and then for forming soft tissue. Such methods involve placing a growth factor at a desired site of a body with use of techniques including resorbable and non-resorbable carriers, gels, time-

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release capsules, and granules. In addition, the growth factor may be placed in the body orally, systemically, by injection, through the respiratory tract, by making an incision in the body and then inserting the growth factor. I note further that the growth factor and/or carrier may be activated by tissue pH, enzymes, ultrasound, electricity, heat, or in vivo chemicals.

5.   It is well known and established in the medical arts that buds are a primordium or, in other words, a rudiment or commencement of an organ. The process of organ formation includes the differential development of cells to form an organ primordium with the resulting formation of soft tissue. Such process of development is called organogenesis. It is also well known and established in the medical arts that the term "soft tissue" includes blood vessels.

In making the above statement in this Paragraph, I am aware of the definitions attached hereto as Exhibit B. Terms included in the above-mentioned definitions are: bud, primordium; organogenesis, and organ. I am also aware of and have considered the definition of "growth factor" as contained in Column 14 of the aforesaid '235 patent.

6.   The materials included in attached Exhibit C evidence that the placement of growth factors in the body of a human results in the formation of a bud with subsequent growth into soft tissue. These materials report work performed by reputable, skilled scientists and reputable organizations in the medical arts. Consequently, I believe that these reports would be recognized as clearly valid by one of ordinary skill in the medical arts because they report the results of scientific tests conducted by competent, disinterested third parties with use of proper scientific controls.

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7. Based upon the materials included in above Paragraphs 4, 5, and 6, it is my opinion that the process of placing a growth factor at a desired site of a human body will produce a bud that will predictably subsequently grow into soft tissue, as described in the '235 patent, using the techniques identified in above Paragraph 4. My further opinion is that when the techniques and angiogenic growth factors described and disclosed in the Elia patent application are used to place such growth factors in a human host, such placement would result in the formation of soft tissue, e.g., blood vessels. My opinion is in accord with the results obtained by the Isner patent (Exhibit C-6) which employed the same angiogenic growth factors and carrier/technique described and disclosed in the Elia patent application.

8. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: ___2/12/2001___   _Wayne H. Finley_
                                                   Wayne H. Finley

EXHIBIT A

CURRICULUM VITAE

CURRICULUM VITAE

PERSONAL INFORMATION

Name: Wayne H. Finley

Birth: Goodwater, AL
April 7, 1927

Social Security Number: 416-28-1334

Home Address: 3412 Brookwood Road
Birmingham, Alabama 35223
Phone: (205) 969-1942
FAX: (205) 969-0266
Email: whfinley@bellsouth.net

Wife: Sara C. Finley, M.D.
Children: Randall W. Finley, M.D.
Sara J. Finley, J.D.

Religion: Deacon
Dawson Memorial Baptist Church

Civic Club: Shades Valley Kiwanis Club
Rotary Club of Birmingham

Business Address: University of Alabama at Birmingham
1720 7th Avenue South, Sparks 420
Birmingham, Alabama 35294

Departments: Pediatrics, Human Genetics
Phone: (205) 975-2342
FAX: (205) 934-1078

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EDUCATION:

Degree	Year	Institution
BS Secondary Ed.	1947	Jacksonville State University Jacksonville, Alabama
MA Secondary Ed.	1950	University of Alabama University, Alabama
MS Biochemistry	1955	University of Alabama Birmingham, AL
PhD Biochemistry	1958	University of Alabama Birmingham, AL
MD	1960	Medical College of Alabama Birmingham, AL

POSTDOCTORAL TRAINING:

Year	Type	Discipline/Institution
1960-61	Internship	Pediatrics University of Alabama Hospital Birmingham, Alabama
1961-62	Traineeship	Medical Genetics NIH Traineeship Institute for Medical Genetics University of Uppsala, Sweden

MILITARY SERVICE:

1945-46	Active Duty, US Army, Enlisted, Infantry (Germany)
1951-53	Active Duty, US Army, Officer, Chemical Corps Faculty, The Chemical Corps School
1946-74	US Army Reserve Presently LTC CmlC-USAR, Ret.

LICENSURE: Alabama, 1961

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BOARD CERTIFICATION:

1958,1960	National Board of Medical Examiners, Parts I, II
1983	American Board of Medical Genetics
1993	Founding Fellow AMA, MD, American College of Medical Genetics

HOSPITAL APPOINTMENTS:

Staff, The Children's Hospital of Alabama, Birmingham, AL.
Staff, University of Alabama Hospitals, Birmingham, AL.
Consultant Staff, Lloyd Noland Hospital, Fairfield, AL.

ACADEMIC APPOINTMENTS: (In reverse chronological order)

Year   **Rank/title**
All appointments were at the University of Alabama at Birmingham

Year	Rank/title
1996-	Professor *Emeritus*
1986-96	Senior Scientist, Center for Health Risk Assessment and Disease Prevention
1981-96	Senior Scientist, Cystic Fibrosis Research Center
1980-96	Adjunct Professor in Biology
1977-96	Professor of Biochemistry
1975-96	Professor of Public Health and Epidemiology
1975-96	Associate Professor Physiology and Biophysics
1975-77	Associate Professor of Biochemistry

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1970-96	Senior Scientist, Associate Member, Comprehensive Cancer Center
1970-96	Professor of Pediatrics**(Primary Appointment)**
1968-75	Assistant Professor of Physiology and Biophysics
1966-70	Associate Professor of Pediatrics
1966-96	Director, Laboratory of Medical Genetics
1965-75	Assistant Professor of Biochemistry
1962-66	Assistant Professor of Pediatrics

AWARDS/HONORS:

Who's Who Among Students in American Colleges and Universities, 1947
Kappa Delta Pi, 1947, and Phi Delta Kappa,1949, Honorary Education Fraternities
McBurney Cup (1960), Sigma Chapter, Phi Beta Pi Medical Fraternity, 1957
Alpha Omega Alpha, Honorary Faculty, 1971
Annual Medical Award (1969), Alabama Association for Retarded Citizens
Outstanding Educators of America, 1971
AMA Physicians Recognition Award, 1971, 1975, 1981, 1984, 1987, 1990, 1993,   1996
 Who's Who in America, 1974
Honorary Member, Alabama Pedodontics Society
Who's Who in Alabama
Personalities of the South, 1972
Omicron Delta Kappa, 1976, Honorary Faculty
Distinguished Medical Alumni Award, 1978, University of Alabama School of
 Medicine Alumni Association
American Men and Women of Science
Who's Who in South and Southwest
Who's Who in Science and Technology

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Turlington Award, Planned Parenthood of Alabama, Inc., 1982
Distinguished Faculty Lecturer, Medical Center, UAB, 1983
Who's Who in Science and Engineering
Wayne H. and Sara Crews Finley Chair in Medical Genetics established UAB,
      1986
Alumnus of the Year, Jacksonville State University, Jacksonville, AL, 1989
Newcomen Society of the United States, 1990
Sat for Portrait, Reynolds Historical Library, UAB, 1991
Fellow, Royal Society of Medicine, 1995
Will Gaines Holmes Award, Children's Aid Society, 1999

PROFESSIONAL SOCIETIES:

American Society of Human Genetics
American Association for the Advancement of Science
American Federation of Clinical Research
American Chemical Society
American Institute of Chemists, Inc.
Society for Experimental Biology and Medicine
The New York Academy of Sciences
Christian Medical Society
Southern Medical Association
Southern Society for Pediatric Research
Medical Association of the State of Alabama
Alabama Academy of Science
Alabama Association for Retarded Citizens
Jefferson County, Alabama Pediatric Society
Jefferson County, Alabama Medical Society
 University of Alabama National Alumni Association
Alumni Association, University of Alabama School of Medicine
Associate, Alabama Chapter, American Academy of Pediatrics
NIH Alumni Association, Bethesda, Maryland
American Medical Association
Southeastern Regional Genetics Group
American College of Medical Genetics, Founding Fellow

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COUNCILS AND COMMITTEES:

1966-67	Committee on Genetic Counseling (ad hoc), Children's Bureau, Department of HEW
1968-70	Chairman, University of Alabama Two-Year Medical Program at Tuscaloosa
1971-72	Special Advisory Committee for Minority Students, University of Alabama in Birmingham
1972-76	Research Committee, Alabama Association for Retarded Citizens
1972-73	President, Sigma Xi, University of Alabama at Birmingham Chapter
1972-77	Subcommittee in Research, Shriners Hospitals for Crippled Children
1973-	Chairman, Carey W. Phillips Travel Fellowship Committee
1973-74	President, Kiwanis Club of Shades Valley, Alabama District
1974-75	President, Alumni Association, University of Alabama School of Medicine
1975-80	Human Use Committee, Biomedical Research, Inc.
1975-77	Maternal and Child Care Committee, Chairman Jefferson County Medical Society
1976-82	Prevention Committee, Chairman, Alabama Association for Retarded Citizens
1977-78	University of Alabama System Medical Education Program, Committee on Continuing Education
1977-80	Member, National Advisory Research Resources Council of the National Institutes of Health, Bethesda, MD
1978-81	Member, Law Center Planning Committee, University of Alabama
1978-81	Member, Board of Censors, Jefferson County Medical Society
1978	Member, Health Issues Coalition, Birmingham Regional Hospital Council
1978-96	Member, Medical Advisory Committee, Central Alabama Chapter, National Multiple Sclerosis Society
1978-80	Member, Board of Directors, Alabama Academy of Science
1978-96	Project Director, Alabama Medical Genetics Program
1981-83	Board of Advisors, Center for Public Law and Service, University of Alabama Law Center, University, Alabama
1981-82	Chairman, Prevention and Research Committee, Association for Retarded Citizens
1981-90	Treasurer, Birth Defect and Clinical Genetics Society, Boston, MA
1981-	Member, American Physiological Society
1981	President-Elect, Jefferson County, Alabama Medical Society
1982	Health Services Committee, Birmingham Chamber of Commerce
1982	Member, New Horizons Marketing Task Force, United Way
1982	Member, Birmingham Steering Committee

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1982-2000	Member, Board of Directors, Southeastern Regional Genetics Group (SERGG), Alabama Representative
1983	Member, Citizens Supervisory Committee
1983-84	President, Jefferson County, Alabama Medical Society
1983-85	Archives Committee, Jefferson County Medical Society
1984	External Reviewer for Graduate Program, Department of Medical Genetics, Indiana University Medical Center
1984	Member, Board of Trustees of the Jefferson County Medical Society
1984-86	President, Caduceus Club, University of Alabama School of Medicine
1984	Member, Research and Education Foundation, BRHC-JCMS.
1984-00	Member, Advisory Committee for MCH Regional Genetics Program.
1984-86	Member, UAB Faculty and Staff Benevolent Council
1984-86	Member, Liaison Committee between JCMS and the Birmingham Regional Hospital Council
1986-95	Member, Promotions Committees, College of Community Health Sciences, The University of Alabama and School of Primary Medical Care, University of Alabama at Huntsville
1987	Committee on Future Needs in Medical Genetics, Genetics Services Branch, Bureau of Health Care Delivery and Assistance, USPHS
1988-90	Sickle Cell Advisory Council, Alabama State Department of Public Health
1988-90	Chairman, Emmett B. Carmichael Award Committee, Alabama Academy of Science
1989-	Member, SOS Foundation of Jefferson County
2000-2001	Chairman, SOS Foundation of Jefferson County
1989-96	Continuing Medical Education Committee, The Children's Hospital of Alabama
1991-	Board of Trustees, Alabama Academy of Science
1991-	Counselor, Medical Association of the State of Alabama
1992-95	Member, The University of Alabama College of Education Steering Committee
1993-99	JSU Foundation Board, Jacksonville State University
1993-97	Member, Education Committee, American College of Medical Genetics
1995	Program Director, 3rd Annual Meeting, American College of Medical Genetics, San Antonio, TX, March 12-14, 1996
1995	External Reviewer, Department of Medical Genetics, Indiana University School of Medicine, Indianapolis, IN
1996-98	Ethics Task Force, Birmingham Regional Council of Ala HA
1997-00	Editor, Southeastern Regional Genetics Group Newsletter
1998-	Alabama Healthcare Hall of Fame Advisory/Nominating Committee

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Graduate Committees - Member

1968	Mancinelli, SA	MS, Physiology/Biophysics
1969	Ciola, B	MS, Dentistry
1970	McDanal, CE, Jr	MS, Basic Medical Science
	Darden, SS	MS, Physiology & Biophysics
	Barham, WW	PhD, Anatomy
1972	Hutto, SC	MS, Physiology/Biophysics
	Hoffman, K	MS, Physiology/Biophysics
1974	Wilkerson, SA	PhD, Physiology/Biophysics
1975	Garrett, JH	MS, Physiology/Biophysics
1978	Michael, EB	PhD, Biochemistry
1979	Watkins, JA, Jr	MS, Biochemistry
1981	Smith, JL	PhD, Physiology/Biophysics
1982	Barganier, CH	DrPH
	Mansson-Rahemtulla, B	MS, Oral Biology
1983	Conary, JT	PhD, Physiology/Biophysics
1984	Yang-Feng, TL	PhD, Biology
1985	Dauzat, EA	MS, Biology
1986	Harman, L	MS, Medical Genetics
1988	Martin, RK	PhD, Medical Genetics
	Hall, TM	MS, Medical Genetics
1989	Nowakowski, R	PhD, Medical Genetics
1991	VanderVegt, FP	PhD, Medical Genetics
	Harman, L	PhD, Medical Genetics
	Han, Jian	PhD, Medical Genetics

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1992	Eipers, P	PhD, Medical Genetics
	Edge, M	PhD, Medical Genetics
	Barnoski, B	PhD, Medical Genetics
1993	Perry, R	PhD, Medical Genetics
	Lyon, E	PhD, Medical Genetics
1994	Crawford, E	PhD, Medical Genetics
1995	Knops, J	PhD, Medical Genetics
	Kelly, L	PhD, Medical Genetics
	Watts, H	Ms, Medical Genetics
1996	Chu, Da-Chang	PhD, Medical Genetics
	Barker, S	MS, Basic Sciences
1966	Tsoumanis, F.	PhD, Medical Genetics
	Rosenfeld, M.	PhD, Medical Genetics
	McGannon, M.	PhD, Medical Genetics
	Li, Peining	PhD, Medical Genetics
1997	Brown, T.	PhD, Medical Genetics

Master's Degrees - Chairman

1968	Mancinelli, Sergio A	MS, Physiology/Biophysics
	Gebhart, Harold E	MS, Basic Medical Science
	Payne, Gillis	MS, Basic Medical Science
	Taylor, Peyton T	MS, Basic Medical Science
1969	Ciola, Benjamin	MS, Dentistry
1970	Jennings, GC	MS, Laboratory Science
1971	Carlson, Robert H	MS, Basic Medical Science
1972	Pederson, Martha I	MS, Physiology/Biophysics
	Vinson, Paula C	MS, Physiology/Biophysics
1973	Luketic, Davor	MS, Physiology/Biophysics
1974	Beatty, Paula J	MS, Biochemistry

1975	Garrett, John H	MS, Physiology/Biophysics
	Honea, Kathryn L	MS, Physiology/Biophysics
1976	Varner, Robert E	MS, Physiology/Biophysics
1977	Ready, James M	MS, Physiology/Biophysics
	Watson, Michael S	MS, Physiology/Biophysics
	Stockard, Cecil R	MS, Biochemistry
1980	Shunnarah, Richard	MS, Physiology/Biophysics
1982	Mihelich, Kristin	MS, Physiology/Biophysics
	Chandler, Walter S	MS, Physiology/Biophysics
1984	Jesse, Mary Ann	MS, Basic Medical Science
1985	Hall, Robin T	MS, Basic Medical Science
1988	Grimm, Karel Jo	MS, Medical Genetics

PhD Degrees - Chairman

1974	McPhee, Hugh T	PhD, Physiology/Biophysics
	Vinson, Paula C	PhD, Physiology/Biophysics
	Wilkerson, Shirley A	PhD, Physiology/Biophysics
1978	Naftel, John P	PhD, Anatomy
	Michael, Edward Barry	PhD, Biochemistry
1979	Carroll, Andrew J	PhD, Physiology/Biophysics
1981	Watson, Michael S	PhD, Physiology/Biophysics
1983	McCombs, Jerome L	PhD, Physiology/Biophysics
1985	Johnson, Evelyn	PhD, Physiology/Biophysics
1989	Warren, Joe	PhD, Medical Genetics
	Wells, Gretchen	PhD, Medical Genetics
1995	John Longshore	PhD, Medical Genetics
1997	Virginia Tanner Thurston	PhD, Medical Genetics

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UNIVERSITY ACTIVITIES:

1966-75	Medical Student Research Day Chairman
1972-74,1983-96	University of Alabama in Birmingham Graduate Council
1973-88	Editorial Board, Alabama Journal of Medical Sciences
1973-74	Chairman, UAB Distinguished Faculty Lectureship Committee
1975-96	Executive Cancer Committee, Medical and Dental Staff, University of Alabama Hospitals
1976-78	Management Committee, Center for Developmental and Learning Disorders
1978-	Associates of the Reynolds Library, University of Alabama in Birmingham
1978-80	Member, Faculty Council, University of Alabama School of Medicine
1978-79	Liaison Committee to the President, University of Alabama in Birmingham
1979-82	Joint Faculty Status Committee of the Schools of Medicine & Dentistry, University of Alabama in Birmingham
1979-80	Member, Grievance Panel, University of Alabama in Birmingham
1981-	Chairman, Reynolds Library Associates Steering Committee, University of Alabama at Birmingham
1983-00	Board of Directors, Southeastern Regional Genetics Group
1983-96	Director, Graduate Program in Medical Genetics
1983-89	Member, Faculty Council, University of Alabama School of Medicine
1984-86	Board of Directors, Greater Birmingham Chamber of Commerce
1985	Connor Essay Prize Committee, University College, UAB
1985-86	American Medical Association Award Program Committee, UAB
1985-87	Chairman, Faculty Council, University of Alabama School of Medicine
1987	Member, Search Committee, UAB Senior VP for Health Affairs1988-
1988-	Member, Marie and Emmett Carmichael Fund for Graduate Students in Biosciences
1992-	Member, UAB Archives Committee
1995-96	Senator, UAB Faculty Senate, Member, Faculty Affairs Committee
1995-96	Faculty Representative to UA Board of Trustees, University of Alabama at Birmingham

GRANT SUPPORT:

REFERENCES

THESIS: **Finley, Wayne H.**: The Inhibition of Mouse Sarcoma 180 by Various Hydroxyalkyl and Thioalkyl-Phthalimides.

DISSERTATION: **Finley, Wayne H.**: Bis-Mesylates: I. Synthesis and Mechanism of Formation. II. Evaluation of Hemopoietic Effects and Inhibition of Mouse Sarcoma 180 and Mouse Ehrlich's Ascites Tumor in Young Mice.

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Gustavson KH, Finley SC, **Finley WH**, Jalling B. 1964. A 4-5/21-22 Chromosomal Translocation Associated with Multiple Congenital Anomalies. Acta Pediat. 53:172-181.

Finley Wayne H, Finley Sara C, Pittman CS. 1964. Phenotypic Male with Mosaic Sex Chromosomes. J Am Med Assoc 188:758-760.

Byrd Wm J, **Finley WH**, Finley WH, Finley SC, McClure Sally. 1964. Antigenic Properties of Mitogenic Factor in Phytohemagglutinin. Lancet 2:420-421.

Finley Sara C, **Finley Wayne H**, Rosecrans Clarence J, Phillips Carey. 1965. Exceptional Intelligence in a Monogolid Child of a Family with a 13-15/Partial 21(D/Partial G) Translocation. New Engl J Med 272:1098-1092.

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Schneyer Charlotte A, **Finley Wayne H**, Finley Sara C. 1967. Increased Chromosome Number of Rat Parotid Cells after Isoproternol. Proc Soc Exp Biol and Med 125:722-728.

Byrd William J, Hare Kendrick, **Finley Wayne H**, Finley Sara C. 1967. Inhibition of the Mitogenic Factor in Phytohaemagglutinin by an Antiserum. Nature 213:622-624.

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Christian ST, Monti JA, **Finley WH**. 1977. Membrane Fluidity in Normal and Cystic Fibrosis Fibroblasts. Biochem Biophys Res Com 79:966-972.

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Howard-Pebbles PN, **Finley WH**. 1983. Screening of Mentally Retarded Males for Macro-orchidism and the Fragile X Chromosome. Am J Med Genet 5:631-635.

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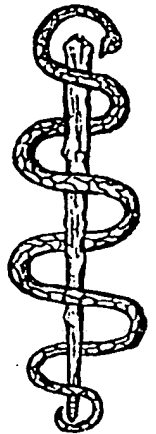
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2/4/2001

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EXHIBIT
B

DEFINITIONS

STEDMAN'S MEDICAL DICTIONARY

ILLUSTRATED

A vocabulary of medicine and its allied sciences, with pronunciations and derivations

TWENTY-SECOND EDITION

Completely revised by a staff of 33 editors, covering 44 specialties and subspecialties

anxiety accompanying psychosomatic disorders; should not be used for nausea of pregnancy.

bud. A structure that resembles the b. of a plant.

bronchial b., one of the outgrowths from the primordial bronchus responsible for the continued ramification of the embryonic bronchial tree.

end b., tail b.

farcy b., one of a number of nodules formed along the course of the subcutaneous lymphatics in cases of glanders.

gustatory b., *caliculus* gustatorius.

liver b., the primordial cellular outgrowth from foregut entoderm of the embryo that gives rise to the parenchyma of the liver.

lung b., in the embryo, one of the two lateral outgrowths from the respiratory primordium that ultimately forms the epithelial portions of the lung.

metanephric b., ureteric b.; the primordial cellular outgrowth from the mesonephric duct that gives rise to the epithelial lining of the ureter, pelvis and calyces of the kidney, and the straight collecting tubules.

syncytial b., syncytial *knot*.

tail b., end b.; the rapidly proliferating mass of cells at the caudal extremity of the embryo.

taste b., *caliculus* gustatorius.

tooth b., the primordial structures from which a tooth is formed; the enamel organ, dental papilla, and the dental sac enclosing them.

ureteric b., metanephric b.

vascular b., an endothelial sprout arising from a blood vessel.

Budd, George, London physician, 1808–1882. See B.'s *cirrhosis, jaundice, syndrome.*

Budde (bood'deh), E., Danish sanitary engineer, *1871. See B. process.*

buddeize (bood'de-ize). To treat by the Budde process.

budding. Gemmation.

Budge (bood'ga), Julius L., German physiologist, 1811–1888. See B.'s *center.*

Budin (bü-dän'), Pierre C., French gynecologist, 1846–1907. See B.'s obstetrical *joint,* B.'s *pelvimeter.*

Buerger, Leo, New York physician, 1879–1943. See B.'s *disease,* Winiwarter-B. *disease,* B.'s *stain.*

bufa-, bufo-. Combining forms that denote origin from toads. They are used in the systematic and trivial names of a great number of toxic substances (genins) isolated from plants and animals containing the bufanolide structure (see bufanolide). Prefixes denoting species origin are often attached, *e.g.,* marinobufagin, marinobufotoxin.

bufagenins. Bufagins.

bufagins. Bufagenins; a group of steroids (bufanolides) in the venom of a family of toads, the Bufonidae, having a digitalis-like action upon the heart (*e.g.,* bufotalin); *cf.* bufotoxins. For structure of bufanolides, see steroids.

bufalin. A specific type of bufanolide, containing 3β,14-dihydroxy-5β,14β-bufa-20,22-dienolide. For structure of bufanolide, see steroids.

bufanolide. Fundamental steroid lactone of several squill-toad (Bufonidae) venoms or toxins; also found in the form of glycosides in plants (*cf.* digitalis). The steroid is essentially that of 5β-androstane, with a 14β-H. The lactone at C-17 is structurally related to —CH(CH₃)CH₂CH₂CH₃ radical attached to C-17 in the cholanes, and is in the same configuration as that of cholesterol (*i.e.,* 20 R); in some species, b. is formed from cholesterol. Various b. derivatives having unsaturation in the lactone ring (20,22) or elsewhere (4) are known as bufenolides (one double bond), bufadienolides (*e.g.,* bufalin, telecinobufagin, marinobufagin, bufogenin B, bufotalin, bufotoxin), bufatrienolides (*e.g.,* scillarenin), etc. They have varying numbers of hydroxyl groups at positions 3, 5, 14, and 16, and these may be further substituted (*e.g.,* bufatalin, bufotoxin, gitoxigenin). For structure, see steroids.

buffer. 1. A mixture of an acid and its conjugate base (salt), such as H₂CO₃/HCO₃⁻; H₂PO₄⁻/H₃PO₄⁻, which when present in a solution reduces any changes in pH that would otherwise occur in the solution when acid or alkali is added to it. Thus the pH of the blood and body fluids is maintained virtually constant (pH 7.45)

although acid metabolites are continually being formed in the tissues and CO₂(H₂CO₃) is lost in the lungs. See also conjugate acid-base pair, under conjugate. 2. To add a b. to a solution and thus give it the property of resisting a change in pH when it receives a limited amount of acid or alkali.

b. capacity, the amount of hydrogen ion (or hydroxyl ion) required to bring about a specific pH change in a specified volume of a b. (see b. value).

b. pair, an acid and its conjugate base (anion).

secondary b., see Hamburger's *law.*

b. value, the power of a substance in solution to absorb acid or alkali without change in pH; this is highest at a pH equal to the pK of the acid of the b. pair (see b. capacity).

b. value of the blood, the ability of the blood to compensate for acid-alkali fluctuations without disturbance of the pH.

Buffon (boo'-fon), Compt de (Georges Louis Leclerc), French naturalist, 1707–1788. Published *Histoire Naturelle.* Some of his views on evolution and the origin of species anticipated Darwin by more than a hundred years.

buffy coat. Crusta inflammatoria; crusta phlogistica; the upper, lighter portion of the blood clot (coagulated plasma and white blood cells), occurring when coagulation is delayed so that the red blood cells have had time to settle a little; the portion of centrifuged, anticoagulated blood which contains leukocytes and platelets.

bufo-. See bufa-.

bufogenin B. A steroid toxin from Chinese toads; a 3β,14,16-trihydroxy-bufa-20,22-dienolide; *cf.* bufalin.

Bufonidae [L. *bufo,* toad]. A family of toads whose dermal glands secrete several kinds of pharmacologically active substances having a cardiac action similar to that of digitalis.

bufotalin. The steroid of a bufotoxin (bufogenin). It is bufogenin B acetylated at the C-16 OH.

bufoten'ine. Mappine; 3-(2-dimethylaminoethyl)indol-5-ol; N,N-dimethylserotin; a psychotomimetic agent isolated from the venom of certain toads. It raises the blood pressure by a vasoconstrictor action and produces psychic effects including hallucinations. It is also present in several plants and is one of the active principles of cohoba.

bufotox'in. Vulgarobufotoxin; a toxic substance in venom of *Bufa vulgaris,* the common European toad; bufotalin esterified with suberylarginine at C-14 OH group.

bufotox'ins. A group of steroid lactones (conjugates of bufogenins and suberylarginine at C-14) of digitalis present in the venoms of the Bufonidae. Their effects are similar to but weaker than those of the bufagins.

bug'gery [O.F. *bougre,* heretic]. Bestiality; sodomy.

Buhl (bool), Ludwig von, German pathologist, 1816–1880. See B.'s *disease.*

Buist, Robert C., Scottish obstetrician, 1860–1939. See B.'s *method.*

bulb [L. *bulbus,* a bulbous root]. 1. Any globular or fusiform structure. 2. *Medulla* oblongata. 3. A short, vertical underground stem of plants such as scilla and allium.

aortic b., *bulbus* aortae.

arterial b., *bulbus* aortae.

carotid b., *sinus* caroticus.

b. of corpus spongiosum, *bulbus* penis.

dental b., the papilla, derived from mesoderm, that forms the part of the primordium of a tooth which is situated within the cup-shaped enamel organ.

duode'nal b., duodenal *cap.*

end b., one of the oval or rounded bodies in which the sensory nerve fibers terminate in mucous membrane.

b. of eye, *bulbus* oculi.

hair b., *bulbus* pili.

ju'gular b., *bulbus* venae jugularis.

Krause's end b., *corpusculum* bulboideum.

b. of lateral ventricle, a rounded elevation in the dorsal part of the medial wall of the posterior horn of the lateral ventricle, produced by the forceps major.

olfac'tory b., *bulbus* olfactorius.

b. of penis, *bulbus* penis.

rachid'ian b., *medulla* oblongata

Rouget's b., a venous pl[...]

taste b., *caliculus* gustat[...]

b. of ure'thra, *bulbus* p[...]

b. of vestibule, *bulbus* v[...]

bulbar. 1. Relating to a bull[...] medulla oblongata.

bulbi'tis. Inflammation of[...]

bulbocap'nine [G. *bolbos,*[...] An alkaloid from *Corydalis Fumariaceae).* Produces a s[...] recommended in the treatmen[...] disease, paralysis agitans, an[...]

bul'bocaverno'sus. See u[...]

bulboid [G. *bolboeidēs,* fr.[...] resemblance]. Bulb-shaped.

bulbonu'clear. Relating to[...]

bulbopon'tine. Denoting t[...] pons and the region of the m[...]

bulbosac'ral. Relating to[...] segments of the spinal cord.

bulbospin'al. Relating to th[...] particularly to nerve fibers in[...]

bulbourethral (bul'bo-u-re'[...]

bulbus, gen. and pl. **bull**[...]

b. aor'tae [NA], aortic bulb[...] dilation where the truncus art[...]

b. cordis, b. aortae.

b. cornus posterior'is [N,[...] lateral ventricle of the brain; [...] of the posterior horn produce[...] of the corpus callosum as the[...] lobe.

b. oc'uli [NA], bulb of the [...] proper without the appendage[...]

b. olfacto'rius [NA], olf[...] anterior extremity of the olfac[...] plate of the ethmoid and rece[...]

Olfactory bulb

Cribriform plate of ethmoid

Olfactory mucosa

Bulbus O[...]

Diagram of olfactory muc[...] y Cajal), showing neuronal rel[...] Copenhaver, W. F.: *Bailey's*[...] The Williams & Wilkins Co.,[...]

b. penis [NA], bulb of corp[...]
b. urethrae; the expanded poster[...] penis lying in the interval betw[...]
b. pili [NA], hair bulb; the lo[...] that fits like a cap over the pap[...] follicle.
b. ure'thrae, b. penis.
b. venae jugula'ris [NA], b[...] dilated parts of the internal jugu[...] at the beginning of the internal j[...]

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Thesaurus

bud[1,noun]
bud[2,verb]
Go To : bud scale

Main Entry: [1]bud
Pronunciation: 'b&d
Function: *noun*
Etymology: Middle English *budde*
Date: 14th century
1 : a small lateral or terminal protuberance on the stem of a plant that may develop into a flower, leaf, or shoot
2 : something not yet mature or at full development: as **a** : an incompletely opened flower **b** : CHILD, YOUTH **c** : an outgrowth of an organism that differentiates into a new individual : GEMMA; *also* : PRIMORDIUM
- **in the bud** : in an early stage of development in the bud>

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For further explanation of these symbols see the Thesaurus Symbol Guide.

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Thesaurus

Main Entry: **pri·mor·di·um**
Pronunciation: -dE-&m
Function: *noun*
Inflected Form(s): *plural* **pri·mor·dia** /-dE-&/
Etymology: New Latin, from Latin
Date: 1671
: the rudiment or commencement of a part or organ

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Dictionary Pronunciation Key

- \&\ as **a** and **u** in **abut**
- \ᵉ\ as **e** in **kitten**
- \&r\ as **ur** and **er** in **further**
- \a\ as **a** in **ash**
- \A\ as **a** in **ace**
- \ä\ as **o** in **mop**
- \au\ as **ou** in **out**
- \ch\ as **ch** in **chin**

- \e\ as **e** in **bet**
- \E\ as **ea** in **easy**
- \g\ as **g** in **go**
- \i\ as **i** in **hit**
- \I\ as **i** in **ice**
- \j\ as **j** in **job**
- \[ng]\ as **ng** in **sing**
- \O\ as **o** in **go**

- \o\ as **aw** in **law**
- \oi\ as **oy** in **boy**
- \th\ as **th** in **thin**
- \th\ as **th** in **the**
- \ü\ as **oo** in **loot**
- \u\ as **oo** in **foot**
- \y\ as **y** in **yet**
- \zh\ as **si** in **vision**

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organogenesis

organogenesis,

in embryology, the series of organized integrated processes that transforms an amorphous mass of cells into a complete organ in the developing embryo. The cells of an organ-forming region undergo differential development and movement to form an organ primordium, or anlage. Organogenesis continues until the definitive characteristics of the organ are achieved. Concurrent with this process is histogenesis; the result of both processes is a structurally and functionally complete organ. The accomplishment of organogenesis ends the period during which the developing organism is called an embryo and begins the period in which the organism is called a fetus. See also histogenesis.

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```
  Thesaurus
     organ
     barrel organ
  Go To  electric organ
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Main Entry: **organ**
Pronunciation: 'or-g&n
Function: *noun*
Etymology: Middle English, partly from Old English *organa,* from Latin *organum,* from Greek *organon,* literally, tool, instrument; partly from Old French *organe,* from Latin *organum;* akin to Greek *ergon* work -- more at WORK
Date: before 12th century
1 a *archaic* : any of various musical instruments; *especially* : WIND INSTRUMENT **b** (1) : a wind instrument consisting of sets of pipes made to sound by compressed air and controlled by keyboards and producing a variety of musical effects -- called also *pipe organ* (2) : REED ORGAN (3) : an instrument in which the sound and resources of the pipe organ are approximated by means of electronic devices (4) : any of various similar cruder instruments
2 a : a differentiated structure (as a heart, kidney, leaf, or stem) consisting of cells and tissues and performing some specific function in an organism **b** : bodily parts performing a function or cooperating in an activity organs>
3 : a subordinate group or organization that performs specialized functions organs of government>
4 : PERIODICAL

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Thesaurus Symbol Key

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EXHIBIT C

EXHIBIT C
SUMMARY OF MATERIALS

EXH. NO.	MATERIAL AND DATE	SOFT TISSUE	TECHNIQUE	GROWTH FACTOR
C-1	Science Daily (American Heart Association), 1998, "Study is first ever to document protein therapy induces creation of new blood vessels to the human heart" SYNOPSIS: For the first time ever, growth factor inserted into the body grows a new vascular system.	Blood vessels to heart	Injection	Human recombinant basic fiberblast growth factor (genetically manipulated and produced)
C-2	Circulation, 1998, "Induction of neoangiogenesis in ischaemic myocardium by human growth factors: first clinical results of a new treatment of coronary heart disease" SYNOPSIS: A new therapeutic concept and followup tests confirm a true de novo vascular system was formed . Vascular buds consisting of endothelial sprouts (capillaries) were created. The capillaries grew further and differentiated into two-layered metarterioles. The process of organogenesis continued with the metarterioles differentiating into three-layered arterioles (arteries).	Blood vessels to heart	Injection	Human recombinant basic fiberblast growth factor (genetically manipulated and produced)

EXH. NO.	MATERIAL AND DATE	SOFT TISSUE	TECHNIQUE	GROWTH FACTOR
C-3	<u>Circulation</u>, 1998, Editorial, "Angiogenic therapy of the human heart" <u>SYNOPSIS</u>: Basic research in a different field (cancer) purified angiogenic growth factors in the 1980's. A novel clinical application of these growth factors introduces a new modality-the regulation of blood vessel growth.	Editorial	Editorial	Editorial
C-4	<u>NIH Press Release</u>: 1999, "Growing New blood vessels with a timed-release capsule of growth factor is a promising treatment for heart bypass patients, finds NHLBI Study" <u>SYNOPSIS</u>: Researchers at Harvard Mecial School inserted timed-release capsules of basic fibroblast growth factor into [human] heart muscle to grow new blood vessels.	Blood vessels to heart	Insertion of timed-release capsule	Basic fibroblast growth factor
C-5	<u>The Lancet</u>, 1996, "Clinical Evidence of angiogenesis after arterial gene transfer of phVEGF in Patient with Ischaemic limb" <u>SYNOPSIS</u>: Growth factor plus living material (plasmid) inserted into the body with a gel carrier to grow new blood vessels in the leg of a patient.	Blood vessels to leg	Balloon Catheter/ hydrogel	Vascular endothelial growth factor plus living material (plasmid)

EXH. NO.	MATERIAL AND DATE	SOFT TISSUE	TECHNIQUE	GROWTH FACTOR
C-6	U.S. Patent No. 5,652,225 (1997) Parent application filed 10/04/94 SYNOPSIS: The formation of new blood vessels in a human host by inserting a growth factor with a carrier into the body.	Formation of new blood vessels	Balloon catheter/ hydrogel	Angiogenic growth factors
C-7	Harvard University Gazette, 1998, "New Arteries Grown in Diseased Hearts" SYNOPSIS: Harvard Medical School researchers inject basic fibroblast growth factor through a carrier (tube) to grow new arteries in a human heart.	Formation of new arteries in hearts	Injection via tube (catheter); and implanted timed-release capsules	Basic fibroblast growth factor

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Source: American Heart Association (http://www.americanheart.org/)

Date: Posted 3/2/1998

Study Is First Ever To Document Protein Therapy Induces Creation Of New Blood Vessels To The Human Heart

DALLAS, Feb. 24 -- For the first time, scientists have published research evidence that recombinant protein therapy can create new blood vessels to increase blood supply to the human heart. The report from German scientists appears in today's Circulation: Journal of the American Heart Association.

FGF-I, a human growth factor obtained through genetic engineering, was used in 20 patients with some form of ischemic or coronary heart disease, which results from blockages in the vessels leading to and from the heart. By injecting the growth factor near the blocked vessels, the scientists were able to induce neoangiogenesis -- the process by which the body can grow its own new capillary network to bypass occluded vessels.

"This capillary network is a true de novo vascular system," says Thomas-Joseph Stegmann, M.D., head of the department of thoracic and cardiovascular surgery at the Fulda Medical Center, Fulda, Germany. "We were able to use the recognized physiological effects of FGF-I to induce neoangiogenesis in the human ischemic heart."

As early as four days after application of FGF-I, the vascular structure around the diseased vessels was completely altered in all 20 of the patients. Like the spokes of a bicycle wheel, the new capillary vessels radiated outward from the point of injection, resulting in a twofold to threefold increase in blood flow to the heart, says the study's lead author.

Researchers found, on average, the ejection fraction of the 20 patients improved from 50.3 percent to 63.8 percent in the three years following the procedure. Ejection fraction measures how much blood leaves the

heart with each beat and indicates how well the left ventricle -- the heart's main pumping chamber -- is functioning.

In follow-up angiographic imaging of the patients, it was clear that the growth factor injection had stimulated the creation of a new vascular system, says Stegmann. Three months after the procedure, he and his colleagues examined angiograms -- X-ray images of the heart -- of both the treated and control (untreated) patients and found that no blockages had formed in the new vessels.

All of the patients who received the FGF-I three years ago are still alive. The scientists report that no negative side effects have been seen in the patients who received the FGF-I.

Elizabeth Nabel, M.D., an American Heart Association board member, has done extensive research in gene and recombinant protein therapy over the past 12 years. She says this new research is encouraging for cardiovascular surgeons.

"It's a very important therapy for patients who have blocked arteries that are not amenable to bypass," says Nabel, professor of internal medicine and physiology and chief, division of cardiology at the University of Michigan. "This is not to say that bypass should be abandoned, but this research shows angiogenesis is a powerful therapy to be used with bypass surgery."

The procedure is still experimental, but scientists say the use of FGF-I may particularly benefit patients whose blocked vessels cannot be treated by cardiac bypass operations.

"At the moment, this procedure could not replace conventional bypass surgery," says Stegmann. "The question remains to be answered whether FGF-I or other growth factors are able to treat occlusions of greater coronary vessels, but currently, this is not possible."

Scientists have used gene therapy to grow vessels in other parts of the body -- such as in the legs in order to improve the health of patients who have blockages in lower leg blood vessels -- but this is the first published account of the use of recombinant protein therapy to induce angiogenesis in human hearts.

FGF-I was obtained from strains of Escherichia coli by genetic engineering, then isolated and highly purified the recombinant FGF-I protein. After several series of animal experiments demonstrated the potency of FGF-I, it was used in humans for the first time.

When scientists create recombinant protein, they take the DNA of a growth factor (in this case FGF-I) and manipulate it into RNA (ribonucleic acid) by growing it in bacteria cultures in the laboratory. RNA is then manufactured into protein, which is isolated and purified

before it is injected into the hearts of patients.

Twenty patients -- 14 men and 6 women who were at least 50 years old -- who had no prior history of heart attack or cardiac surgery had an operation to clear blockages in more than one vessel. All of them had stenosis -- narrowed blood flow due to atherosclerosis -- in their internal mammary artery/left anterior descending coronary artery. During the operative procedure, the growth factor protein -- in a dosage of 0.01 milligrams per kilogram of body weight -- was directly injected into the heart muscle near the blockage.

Prior to using the treatment in humans, the scientists performed several series of animal experiments, most specifically in ischemic rat hearts. Having found that the FGF-I injection worked in those animal models, the researchers theorized that it would also work in humans.

Study co-authors are P. Pecher, M.D.; B.U. von Specht, M.D. and B. Schumacher, M.D.

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Induction of Neoangiogenesis in Ischemic Myocardium by Human Growth Factors

First Clinical Results of a New Treatment of Coronary Heart Disease

B. Schumacher, MD; P. Pecher, MD; B.U. von Specht, MD; Th. Stegmann, MD

Background—The present article is a report of our animal experiments and also of the first clinical results of a new treatment for coronary heart disease using the human growth factor FGF-I (basic fibroblast growth factor) to induce neoangiogenesis in the ischemic myocardium.

Methods and Results—FGF-I was obtained from strains of *Escherichia coli* by genetic engineering, then isolated and highly purified. Several series of animal experiments demonstrated the apathogenic action and neoangiogenic potency of this factor. After successful conclusion of the animal experiments, it was used clinically for the first time. FGF-I (0.01 mg/kg body weight) was injected close to the vessels after the completion of internal mammary artery (IMA)/left anterior descending coronary artery (LAD) anastomosis in 20 patients with three-vessel coronary disease. All the patients had additional peripheral stenoses of the LAD or one of its diagonal branches. Twelve weeks later, the IMA bypasses were selectively imaged by intra-arterial digital subtraction angiography and quantitatively evaluated. In all the animal experiments, the development of new vessels in the ischemic myocardium could be demonstrated angiographically. The formation of capillaries could also be demonstrated in humans and was found in all cases around the site of injection. A capillary network sprouting from the proximal part of the coronary artery could be shown to have bypassed the stenoses and rejoined the distal parts of the vessel.

Conclusions—We believe that the use of FGF-I for myocardial revascularization is in principle a new concept and that it may be particularly suitable for patients with additional peripheral stenoses that cannot be revascularized surgically. (*Circulation.* 1998;97:645-650.)

Key Words: growth substances ■ angiogenesis ■ coronary disease

For the cardiac surgeon who is attempting to treat CHD, the use of sections of autologous blood vessels as bypass material is subject to severe limitations. Autologous arterial conduits are in short supply, and segments of the saphenous vein do not remain patent for very long.[1,2] Furthermore, "complete" revascularization is limited if diffuse coronary arteriosclerosis is present and extensive, especially if there are additional peripheral stenoses.

In the search for alternative and/or additional treatment for improving the long-term prognosis, especially in diffuse CHD, attention has recently been directed toward natural angiogenesis.[3,4] Growth factors, especially FGF-I, have recently become of major importance because they can induce angiogenesis.[4,10-12]

Gimenez-Gallego et al[13] succeeded in elucidating the biochemical structure of FGF-I in 1985. Jaye et al[14] isolated human FGF-I from brain tissue in 1986. In 1991, Forough and coworkers[15] successfully used the technique of gene transfer to introduce the information for expressing human FGF-I into apathogenic *Escherichia coli.*

Our aim was to evaluate the information currently available on the biological effect of angiogenetic growth factors in animals and, if appropriate, to use human growth factor for the treatment of CHD. This involved (1) the production of human growth factor by genetic engineering, followed by its isolation, characterization, and purification; (2) using animal experiments to establish its angiogenetic potency and to exclude any possible pathogenic effect; and (3) using FGF-I clinically as an adjunct to coronary surgery and to demonstrate neoangiogenesis in the ischemic human myocardium.

Methods

Production and Purification of FGF-I

The production and purification of human FGF-I is a biochemically elaborate technique. The individual experimental steps have been reported elsewhere.[4,7]

Genetic engineering was used to produce human FGF-I from apathogenic strains of *E coli,* a plasmid containing the genetic information being introduced into the microorganisms.[15] These were kindly provided by Prof T. Maciag (Laboratory of Molecular Biology, American Red Cross, Rockville, Md). After production, FGF-I was eluted by heparin sepharose column chromatography, and several elution fractions were collected and purified by dialysis. Positive protein elution fractions were identified in the BIO-RAD assay[7] by SDS-PAGE,[16] and the biochemical isolation of FGF-I was confirmed by the Western blot method.[17] Further purification was obtained by HPLC.[18] The factors were lyophilized and stored at −32°C and diluted to 1 mL with NaCl solution containing 500 IU of heparin.

EXHIBIT C-2

Selected Abbreviations and Acronyms

CHD = coronary heart disease
EDP = electronic data processing
FGF = basic fibroblast growth factor
HPLC = high-pressure liquid chromatography
IMA = internal mammary artery
LAD = left anterior descending coronary artery

Chorioallantoic Membrane Assay

This established method, which provides a direct demonstration of the effect of growth factors on living tissue, was used to investigate the angiogenetic effect of FGF-I.[19,20] The growth of the allantoic systems can be directly observed by light microscopy. After incubation of 20 fertilized hen eggs for 13 days, the growth factor was applied to the membrane and covered with tissue culture coverslips. Four days later, the membrane was examined under the light microscope and directly compared with controls untreated with FGF-I or treated with heat-denatured FGF-I (70°C for 3 minutes).

Exclusion of the Pyrogenicity of FGF-I

Varying concentrations of FGF-I (0.01, 0.5, or 1.0 mg/kg body weight) were injected subcutaneously, intramuscularly, or intravenously into 27 New Zealand White rabbits, the solvent alone being used for an additional 13 controls. Thereafter, the rectal temperature was taken every half hour for 3 hours, hourly for the rest of the day, and every 8 hours for 12 days. A daily white cell count was also repeated for 12 days (see "Results"). In addition to this, the erythrocyte sedimentation rate and the C-reactive protein values were determined on the 3rd, 6th, 9th, and 12th days after the injection.

Confirmation of the Angiogenetic Potency of FGF-I in Animal Experiments

Supplementary to our earlier experiments,[4,7] the effect of FGF-I was also investigated in the ischemic hearts of inbred Lewis rats (a total of 275 animals, including 125 controls treated with heat-denatured FGF-I, 70°C for 3 minutes). The pericardium was opened via the abdominal wall and diaphragm, and two titanium clips were inserted at the apex of the left ventricle to induce myocardial ischemia. Growth factor (mean concentration of 10 µg) was then injected locally into the site. The coronary vessel system was imaged by aortic root angiography after 12 weeks and, finally, a specimen from the same myocardial region was evaluated histologically.

Clinical Use of FGF-I in Patients With CHD

This study was approved by the Medical Research Commission at the Phillips University of Marburg on August 10, 1993 (No. 47/93). This is the usual ethics commission for our hospital. Twenty patients without any history of infarction or cardiac surgery (14 men and 6 women; minimum age, 50 years) were subjected to an elective bypass operation for multivessel coronary heart disease. The growth factor was applied directly during the operation. As a control group, 20 patients who underwent the same procedure were given heat-denatured FGF-I (70°C for 3 minutes). The choice of treatment was completely random, the names being placed in sealed envelopes and selected in a blinded manner.

The details, nature, and aims of this procedure were explained beforehand to every patient who underwent the operation. In all cases, we received their fully informed consent. Both groups of patients were closely comparable with regard to clinical symptoms, accompanying disorders, cardiovascular risk factors, ventricular function, sex, and age. A comparable coronary morphology was found in both groups.

All patients had a further stenosis in the distal third of the LAD or at the origin of one of its branches in addition to a severe proximal stenosis. The mean ejection fraction of the left ventricle for all patients was 50%. The operative procedure for coronary revascularization with autologous grafts (an average per patient of 2 to 3 venous bypasses and 1 from the left IMA) was routinely performed. FGF-I (mean concen-

Figure 1. Intraoperative administration of growth factor.

tration, 0.01 mg/kg body weight) was injected into the myocardium, distal to the IMA/LAD anastomosis and close to the LAD, during the maintenance of the extracorporeal circulation and after completion of the distal anastomoses (Fig 1). In the control group, heat-denatured FGF-I was substituted for FGF-I. After 12 weeks, the IMA bypasses of all the patients were imaged selectively by transfemoral, intra-arterial, and digital subtraction angiography.

Angiograms obtained in this way were evaluated by means of EDP-assisted digital gray-value analysis, a universally recognized and well-established technique for demonstrating capillary neoangiogenesis.[21-26] Sites of interest both with and without FGF-I (meaning heat-denatured FGF-I) were selected in the vessels filled with contrast medium and in regions of the myocardium distal to the IMA/LAD anastomosis. One hundred pixels were selected from each site of interest and analyzed digitally. Complete blackening of the x-ray films was rated with a gray value of 150, and areas without blackening of the film were allotted a zero value. During the first 5 postoperative days, separate laboratory checks in addition to the routine postoperative follow-up procedures were made twice daily, and the temperature checked three times a day.

Results

After separation, purification, and stabilization, we were able to isolate human FGF-I in all 40 bacterial cultures and demonstrate its high degree of purity. Fig 2 shows an HPLC profile of the growth factor after routine purification. The peak values at the beginning and end of the profile represent impurities that could be identified as E coli proteins. FGF-I could be further separated by fractionated collection, and the control HPLC (Fig 3) merely shows the peak value of this fraction on an otherwise even baseline.

Figure 2. HPLC profile before high purification. HBGF-I indicates human FGF-I; %E, extinction.

Figure 3. HPLC profile after high purification. HBGF-I indicates human FGF-I; %E, extinction.

In the chorioallantoic membrane assay, the angiogenetic potency of FGF-I could be demonstrated in vivo. As early as 4 days after application of the factor, the vascular structure of the membrane was completely altered. Emanating radially from the site of application, an unequivocal growth of new vessels from the original host vessels had grown out into the periphery (Fig 4A). These structures were completely absent from the control group, and a normally developed reticular vascular pattern could be discerned (Fig 4B).

Pyrogenic effects of the human growth factor produced in this way could be definitively ruled out in the animal model. There was no significant rise of body temperature when checked at short intervals and no trace of an inflammatory reaction in comparison with the control group (n=13) in any of the 27 test animals during the period of observation. This result was independent of the concentration and the route of administration (intravenous, subcutaneous, or intramuscular) of the factor.

Earlier investigations into the application of FGF-I to the nonischemic rat heart made it possible to demonstrate neoangiogenesis both histologically and angiographically after 9 weeks in 11 of 12 test animals after the implantation of a tissue bridge pretreated with growth factor between the heart and thoracic aorta. In the control group without FGF-I (n=6), no signs of induced neoangiogenesis could be found.[4,7]

Unequivocal proof of induced neoangiogenesis was also found in the ischemic rat heart. In the test animals, in which myocardial ischemia had previously been induced with titanium clips and growth factor had subsequently been injected into the myocardium, a manifest accumulation of contrast medium was shown by aortic angiography at the site of the FGF-I injection 12 weeks later (Fig 5A), whereas such an accumulation of contrast medium did not appear in any of the control animals (Fig 5B). Histological examination of the myocardium revealed a threefold increase in the capillary density per square millimeter around the site of the FGF-I injection.

When the growth factor FGF-I was used clinically for the first time on the human heart, neoangiogenesis together with the development of a normal vascular appearance could be demonstrated angiographically, exactly as in the earlier animal experiments.[4,7] Selective imaging of the IMA bypasses by intra-arterial digital subtraction angiography confirmed the following result in all 20 patients: at the site of injection and in the distal areas supplied by the LAD, a pronounced accumulation of contrast

10 ng HBGF-I

without HBGF-I

Figure 4. A, Chorioallantoic membrane assay with application of the growth factor. B, Chorioallantoic membrane assay of the control group. HBGF-I indicates human FGF-I.

medium extended peripherally around the artery for ~3 to 4 cm, distal to the IMA/LAD anastomosis (Fig 6A). In the control angiograms of patients to whom only heat-denatured FGF-I had been given, the IMA/LAD anastomosis was also recognizable, but the accumulation of contrast medium described above was absent (Fig 6B). The angiograms of both the treated and control groups were recorded at a rate of four images per second, and these show

10 µgHBGF-I

10 µg/kg HBGF-I

without HBGF-I

without HBGF-I

Figure 5. A, Administration of the growth factor in ischemic rat heart with a clearly discernible accumulation of contrast medium at the site of injection. B, No discernible accumulation of contrast medium in the control group. HBGF-I indicates human FGF-I.

Figure 6. A, Angiography after injection of the growth factor into the human heart shows a pronounced accumulation of contrast medium compared with the control group. B, Angiography in the control group does not show any increased accumulation of contrast medium around the IMA/LAD anastomosis. HBGF-I indicates human FGF-I.

Figure 7 branch c newly gr by newly filled thr

comparable distances between the beginning of the injection and visualization of the medium.

At the site of injection of the FGF-I, a capillary network could be seen sprouting out from the coronary artery into the myocardium. This enabled retrograde imaging of a stenosed diagonal branch to be performed (Fig 7A). Such "neocapillary vessels" can also provide a collateral circulation around additional distal stenoses of the LAD (Fig 7B) and bring about

retrograde filling of a short segment of the artery distal to the stenosis. In none of the angiograms of the treated patients taken 12 weeks after the operation were any new stenoses of the LAD detectable.

The results of EDP-assisted digital gray value analysis for quantification of the neoangiogenesis (Fig 8) gave a mean gray value of 124 for the vessels. The control myocardium reached

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Figure 8. Quantitative gray value analysis of contrast medium accumulation in the angiography shows a twofold to threefold increase in the local blood flow at the site of injection. HBGF-I indicates human FGF-I.

conditions, and this naturally leads to the rapid growth of new capillaries and other blood vessels. Such a physiological process occurs in the development of the placenta, in fetal growth, and in wound healing, as well in the formation of collaterals in response to tissue ischemia. "Angiogenetic growth factors," which are biochemically polypeptides, are essential for such processes as capillary growth or neoangiogenesis. These growth factors (for instance, the human heparin-binding FGF-I) bring about their effect by significantly increasing cell proliferation, differentiation, and migration via a high-affinity receptor system on the surfaces of the endothelial cells.[8,10-12]

During the last few years, several working groups have been able to establish indications for the effective use of growth factors to improve blood flow in the presence of tissue ischemia in animal experiments.[3,9,27] Yanagisawa-Miwa et al[9] succeeded in demonstrating a significant collateralization together with reduction in the size of the infarct after intracoronary administration of growth factor in rabbits. Baffour et al[3] also observed a significant formation of collaterals in ischemic extremities after growth factor administration in animals. Albes et al[27] produced a distinct improvement in the blood flow in ischemic tracheal segments implanted subcutaneously in rabbits by injecting growth factor-enriched fibrin glue locally.

After growth factor was injected into the ischemic rat heart,[4,7] we were able to observe induced neoangiogenesis and confirm it angiographically. We were also able to prove histologically that this neoangiogenesis brings about the development of new vascular structures with a three-layered vessel wall. Angiographic imaging confirmed that these are anatomically normal capillaries and other blood vessels.

The production of human FGF-I by our molecular biological method has proved to be a complex but readily reproducible procedure. From the bacterial cultures, we are able to isolate the factor as a pure substance in sufficient quantities. By in vitro assay and as a result of extensive animal experiments, we were able to exclude the possible pyrogenic effects of FGF-I.

In earlier animal experiments,[4] we were able to demonstrate the proliferative and mitogenic effects of the growth factor on human saphenous vein endothelial cells. Endothelial cell cultures with added growth factor induced a confluent monolayer after only 5 to 9 days, whereas the monolayer was not complete before 7 to 11 days in the control group. In addition to determining the total cell count with a cell counter, we also confirmed this result by analyzing the rate of DNA synthesis by measuring the incorporation of ^{3}H-thymidine into the endothelial cell nuclei using the

10 µg/kg HBGF-I

10 µg/kg HBGF-I

Figure 7. A, Collateralization of stenoses (arrow): a diagonal branch occluded just distal to its origin is filled through the newly grown capillaries. B, Collateralization of stenoses (arrow) by newly grown capillaries: the peripherally stenosed LAD is filled through these vessels. HBGF-I indicates human FGF-I.

a gray value of only 20, and that of the myocardium injected with FGF-I gave a value of 59 (Fig 8).

Discussion

Normal capillaries have a cell population with a low turnover rate of months or years. On occasion, however, a high turnover rate of this cell population is possible even under physiological

method of Klagsbrun and Shing.[28] The cell proliferative potency of FGF-I could be further intensified by adding heparin, a glycosaminoglycan protecting the growth factor from inactivation by cellular enzymes and from heat and chemical denaturation.[29]

On the basis of these in vitro and in vivo experiments, we established for the first time the efficacy of FGF-I for the treatment of CHD, and were able to demonstrate that it can induce neoangiogenesis in situ in the ischemic human heart. This possibility has been widely discussed for many years but never before attempted.

A dense capillary network appeared around the site of injection of the factor in the myocardium of all our treated patients. This capillary network is a true de novo vascular system. Emerging from the proximal segment of the LAD, it sprouts out into the surrounding myocardium, bringing about a twofold to threefold increase in the local blood supply through these newly formed functional vessels. We were able to use the recognized physiological effects of FGF-I (as they occur in the repair mechanism of wound healing or in collateralization of ischemic tissue) to induce neoangiogenesis in the human ischemic heart.

We also consider that administration of FGF-I (produced in this way by genetic engineering), combined with operative myocardial revascularization, may well be an especially appropriate treatment for patients with additional peripheral stenoses that cannot be treated surgically.

In our opinion, neoangiogenesis induced by FGF-I opens up new possibilities for the treatment of ischemic myocardial disease. Furthermore, it could become a new therapeutic concept in the management of diffuse CHD after alternative methods of administration have also been developed. This method of inducing neoangiogenesis is also conceivable as a therapeutic option in other regions of the cardiovascular system in which arterial occlusion has led to ischemia.[30] However, before any such possibilities are realized, many more clinical investigations will have to be performed.

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Angiogenic Therapy of the Human Heart

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The field of angiogenesis research was initiated 27 years ago by a hypothesis that tumors are angiogenesis-dependent.[1] Shortly thereafter, in the early 1970s, it became possible to passage vascular endothelial cells in vitro for the first time.[2] Bioassays for angiogenesis were developed subsequently throughout that decade. The early 1980s saw the purification of the first angiogenic factors.[3-6] By the mid-1980s, angiogenesis inhibitors began to be discovered.[7-9] Translation of these laboratory findings to clinical application started in 1989, when interferon alfa was first used for the treatment of life-threatening hemangiomas in infants.[10-12]

Clinical applications of angiogenesis research are being pursued along three general lines: (1) prognostic markers in cancer patients,[13,14] (2) antiangiogenic therapy (for review, see Reference 15), and (3) angiogenic therapy. The first angiogenic therapy of ischemic vascular disease was the administration of vascular endothelial growth factor (VEGF)/vascular permeability factor to patients with severe peripheral vascular disease in the lower limbs.[16]

In a landmark paper, Schumacher and colleagues now report the first angiogenic therapy of human coronary heart disease.[17] It is an important study, not only because the authors describe how they produced their own recombinant human fibroblast growth factor-1 (FGF-1, also called acidic fibroblast growth factor) and tested it in vitro and in vivo but also because they conducted a randomized controlled clinical trial. In 20 patients with three-vessel coronary artery disease who underwent two or three venous bypass grafts and one from the internal mammary artery, the angiogenic protein FGF-1 was injected into the myocardium close to the left anterior descending coronary artery and distal to its anastomosis with the internal mammary artery. FGF-1 was injected during extracorporeal surgery and again after completion of the anastomosis. Transfemoral, intra-arterial digital subtraction angiography 12 weeks later showed coronary artery neovascularization extending out from the area of FGF-1 injection. Stenoses distal to the anastomosis were bridged by neovascularization. This was similar to the neovascularization observed by the authors in rat hearts injected with FGF-1. Histological sections of rat myocardium showed a threefold increase in microvessel density. In 20 patients undergoing similar coronary artery bypass surgery in whom inactivated FGF-1 was injected, there was no evidence of myocardial neovascularization on the 12-week angiogram.

An advantage of this approach is that it induces local angiogenesis and appears to avoid high levels of circulating angiogenic activity that could possibly stimulate plaque angiogenesis and secondary plaque growth. Why does neovascularization persist for at least 12 weeks after only a single set of intramyocardial injections of the angiogenic protein? Perhaps persistent neovascularization was facilitated by upregulation of VEGF and its receptors in hypoxic tissue.[18] Furthermore, basic FGF and VEGF are synergistic mitogens for endothelial cells in vitro.[19,20] Also, FGF can increase expression of (or mobilize) VEGF.[21]

This report uses primarily anatomic studies to demonstrate increased myocardial neovascularization after angiogenic therapy. We look forward to the follow-up of these patients to learn whether they have significant functional improvement compared with the control group of patients who received inactive FGF. It may be difficult to discriminate the extent to which functional improvement is due to the angiogenic therapy per se, despite use of a control group, because of the concomitant internal mammary artery anastomosis and the relatively small number of patients in this study. Nevertheless, the angiographic documentation of myocardial revascularization suggests that functional improvement should follow.

Although major therapeutic advances in cardiology have been based on the general principles of control of blood pressure, regulation of cardiac rhythm, enhancement of myocardial contractile strength, increased diameter of narrowed coronary arteries, and lysis of intravascular thromboses, the report by Schumacher et al introduces a new modality, the regulation of blood vessel growth. If angiogenic therapy of the myocardium continues to live up to its potential as indicated by this report, we may witness novel refinements in future years as the molecular biology of endothelial cell and smooth cell growth is gradually uncovered. For example, the therapeutic induction of coronary arterial collaterals may someday be optimized by administration of appropriate mixtures of molecules that target different components of the vasculature, ie, the FGFs are mitogenic for vascular endothelial cells and smooth muscle, VEGF[22] is mitogenic primarily for endothelial cells, angiopoietin-1 mediates the recruitment of smooth muscle cells to the wall of new vessels,[23] and angiopoietin-2 appears to prevent or downregulate smooth muscle apposition to the walls of microvessels.[24] It is interesting that the methodology to discover these different vascular cell growth proteins emerged largely from investigations of mechanisms of tumor angiogenesis in studies funded primarily by the National Cancer Institute over many years. The report by Schumacher et al illustrates how unpredictable are the clinical applications that may arise from basic research in a different field.

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KEY WORDS: Editorials ■ angiogenesis ■ growth substances

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EXHIBIT C-4

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Growing New Blood Vessels with a Timed-Release Capsule of Growth Factor is a Promising Treatment for Heart Bypass Patients, Finds NHLBI Study

By The National Heart, Lung, and Blood Institute

Heart bypass patients treated with a timed-release capsule of a substance that promotes the growth of new blood vessels showed evidence of improved blood supply and heart function, according to a study supported by the National Heart, Lung, and Blood Institute (NHLBI) of the National Institutes of Health.

"Growing" blood vessels, a strategy called angiogenesis, is a promising experimental treatment for blocked arteries in bypass surgery patients for whom surgery alone would not adequately restore blood flow to the heart.

Dr. Michael Simons and colleagues at Harvard Medical School inserted timed-release capsules of basic fibroblast growth factor (bfGF) into the heart muscle of patients scheduled for bypass surgery. Patients received either a 10 microgram (mcg) or 100 mcg dose

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of the substance. Other patients
received a harmless placebo capsule
at the time of surgery. The relatively
small study (24 patients total) was
designed to test the safety and
effectiveness of the procedure.

The study, published in the
November 2, 1999 issue of
Circulation, found that there were
no serious adverse effects of the
treatment. Both magnetic resonance
imaging (MRI) and nuclear stress
testing were used to evaluate
changes in blood flow. Stress tests
showed a worsening of blood flow in
the placebo group, no change in the
10 mcg. group and significant
improvement in patients receiving
100 mcg. MRI results showed clear
improvement in blood flow in
patients given 100 mcg. Patients in
the highest dose group were free of
angina (chest pain) but some
patients in the placebo and low-dose
group experienced chest pain.

Simons and colleagues note that a
larger (Phase II) multi-center study
of this approach is currently
underway.

The National Heart, Lung, and Blood
Institute of The National Institutes of
Health. Press Release: **Growing New
Blood Vessels with a Timed-Release
Capsule of Growth Factor is a
Promising Treatment for Heart Bypass
Patients, Finds NHLBI Study**. November
1, 1999. (Online)
http://www.nih.gov/news/pr/nov99/nhlbi-01.htm

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(1 of 1)

United States Patent	5,652,225
Isner	July 29, 1997

Methods and products for nucleic acid delivery

Abstract

The present invention provides a method for the delivery of a nucleic acid to an arterial cell comprising contacting the cell with a hydrophilic polymer incorporating the nucleic acid. The nucleic acid may be any nucleic acid, including antisense DNA or RNA. The nucleic acid may encode hormones, enzymes, receptors or drugs of interest. The nucleic acid is selected based upon the desired therapeutic outcome. For example, in the treatment of ischemic diseases, one would select a DNA encoding an angiogenic protein. The nucleic acid may be carried by a microdelivery vehicle such as cationic liposomes and adenoviral vectors. DNA encoding different proteins may be used separately or simultaneously.

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Appl. No.: **675523**

Filed: **July 3, 1996**

U.S. Class:	514/44; 604/51; 604/52; 604/53; 536/23.5; 536/23.51; 435/320.1; 435/172.1; 435/172.3; 935/9; 935/22; 935/32; 935/33; 935/34; 935/52; 935/57; 424/93.2
Intern'l Class:	A01N 047/40
Field of Search:	514/44 604/51,52,53 536/23.5,23.51 435/320.1,172.1,172.3,235.1,240.2 935/9,22,32,33,34,52,57 424/93.2

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Primary Examiner: Low; Christopher S. F.
Attorney, Agent or Firm: Conlin; David G. Resnick; David S. Dike, Bronstein, Roberts & Cushman, LLP

Parent Case Text

This is a continuation of application Ser. No. 08/318,045 filed on Oct. 4, 1994 now abandoned.

Claims

1. A method for inducing the formation of new blood vessels in a desired target tissue in a human host, comprising contacting an arterial cell in an artery or blood vessel via a balloon catheter coated with a hydrogel polymer admixed with a first DNA encoding an angiogenic protein selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor .alpha. and .beta., platelet-derived endothelial

growth factor, platelet-derived growth factor, tumor necrosis factor .alpha., hepatocyte growth factor and insulin like growth factor and having an operably linked secretory signal sequence or a first DNA encoding a modified angiogenic protein selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor .alpha. and .beta., platelet-derived endothelial growth factor, platelet-derived growth factor, tumor necrosis factor .alpha., hepatocyte growth factor and insulin like growth factor having an operably linked secretory signal sequence, wherein said anglogenic protein induces new blood vessel formation when expressed in said target tissue in an amount effective to induce new blood vessel formation.

2. The method of claim 1, wherein the angiogenic protein is vascular endothelial growth factor.

3. The method of claim 1, wherein the hydrogel polymer is selected from the group consisting of polycarboxylic acids, cellulosic polymers, gelatin, polyvinylpyrrolidone, maleic anhydride polymers, polyamides, polyvinyl alcohols, and polyethylene oxides.

4. The method of claim 1, wherein the hydrogel polymer is a polyacrylic acid polymer.

5. The method of claim 1, wherein the hydrogel polymer is admixed with a second DNA encoding an angiogenic protein selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor .alpha. and .beta., platelet-derived endothelial growth factor, platelet-derived growth factor, tumor necrosis factor .alpha., hepatocyte growth factor and insulin like growth factor and having an operably linked secretory signal sequence or a second DNA encoding a modified angiogenic protein selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor .alpha. and .beta., platelet-derived endothelial growth factor, platelet-derived growth factor, tumor necrosis factor .alpha., hepatocyte growth factor and insulin like growth factor having an operably linked secretory signal sequence, wherein said angiogenic protein induces new blood vessel formation when expressed in said target tissue in an amount effective to induce new blood vessel formation, and wherein said second DNA is not the same as said first DNA.

6. A method for inducing the formation of new blood vessels in a desired target tissue in a human host, comprising contacting an arterial cell in an artery or blood vessel via a balloon catheter coated with a hydrogel polymer admixed with DNA encoding vascular endothelial growth factor and which is expressed in an amount effective to induce new blood vessel formation.

Description

FIELD OF THE INVENTION

The present invention relates to delivery of nucleic acid to arterial cells and compositions therefor.

BACKGROUND OF THE INVENTION

Work from several laboratories (Nabel, et al., Science, 249:1285-1288 (1990); Lim, et al., Circulation, 83:2007-2011 (1991); Flugelman, et al., Circulation, 85:1110-1117 (1992); Leclerc, et al., J. Clin. Invest., 90:936-944 (1992); Chapman, et al., Circ. Res., 71: 27-33 (1992); Riessen, et al., Hum. Gene Ther., 4: 749-758 (1993); and Takeshita, et al., J. Clin. Invest., 93:652-661 (1994), has demonstrated

that recombinant marker genes could be transferred to the vasculature of live animals.

Gene delivery systems employed to date have been characterized by two principal components: a macrodelivery device designed to deliver the DNA/carrier mixture to the appropriate segment of the vessel, and microdelivery vehicles, such as liposomes, utilized to promote transmembrane entry of DNA into the cells of the arterial wall. Macrodelivery has typically been achieved using one of two catheters initially developed for local drug delivery: a double-balloon catheter, intended to localize a serum-free arterial segment into which the carrier/DNA mixture can be injected, or a porous-balloon catheter, designed to inject gene solutions into the arterial wall under pressure. Jorgensen et al., Lancet 1:1106-1108, (1989); Wolinsky, et al., J. Am. Coil. Cardiol., 15:475-485 (1990); March et al., Cardio Intervention, 2:11-26 (1992)); WO93/00051 and WO93/00052.

Double balloon catheters are catheters which have balloons which, when inflated within an artery, leave a space between the balloons. The prior efforts have involved infusing DNA-containing material between the balloons, allowing the DNA material to sit for a period of time to allow transfer to the cells, and then deflating the balloons, allowing the remaining genetic material to flush down the artery. Perforated balloons are balloons which have small holes in them, typically formed by lasers. In use, fluid containing the genetic material is expelled through the holes in the balloons and into contact with the endothelial cells in the artery. These gene delivery systems however, have been compromised by issues relating to efficacy and/or safety.

Certain liabilities, however, inherent in the use of double-balloon and porous balloon catheters have been identified. For example, neither double-balloon nor porous balloon catheters can be used to perform the angioplasty itself. Thus, in those applications requiring both angioplasty and drug delivery, e.g., to inhibit restenosis, two procedures must be preformed. Additionally, the double balloon typically requires long incubation times of 20-30 min., while the high-velocity jets responsible for transmural drug delivery from the porous balloon catheter have been associated with arterial perforation and/or extensive inflammatory infiltration (Wolinsky, et al., J. Am. Coil. Cardiol., 15:475-481 (1990).

SUMMARY OF THE INVENTION

It has now been discovered that nucleic acids can be delivered to cells of an artery or blood vessel by contacting the cells with a hydrophilic polymer incorporating the nucleic acid, thus avoiding the use of a double-balloon or porous balloon catheter and the problems associated with such delivery systems. It has also been demonstrated that, unexpectedly, the percentage of transduced arterial cells is significantly higher using the present invention compared with use of a double-balloon catheter.

By "arterial cells" is meant the cells commonly found in mammalian arteries, including endothelial cells, smooth muscle cells, connective tissue cells and other cells commonly found in the arterial structure.

By "nucleic acid" is meant DNA and RNA, including antisense DNA or RNA.

It has further been discovered that a DNA encoding an angiogenic protein (a protein capable of inducing angiogenesis, i.e., the formation of new blood vessels), delivered by the method of the present invention is expressed by the arterial cell and induces angiogenesis in tissues perfused by the treated blood vessels. This allows for the treatment of diseases associated with vascular occlusion in a variety of target tissues, such as limb ischemia, ischemic cardiomyopathy, myocardial ischemia, cerebral ischemia and portal hypertension.

The present invention provides a method for the delivery of a nucleic acid to an arterial cell comprising contacting the cell with a hydrophilic polymer incorporating the nucleic acid. The nucleic acid may be any nucleic acid, DNA and RNA, including antisense DNA or RNA. The DNA may encode hormones, enzymes, receptors or drugs of interest. The nucleic acid is selected based upon the desired therapeutic outcome. For example, in the treatment of ischemic diseases, the genetic material of choice is DNA encoding an angiogenic protein. The nucleic acid may be carried by a microdelivery vehicle such as cationic liposomes and adenoviral vectors. DNA encoding different proteins may be used separately or simultaneously.

The hydrophilic polymer is selected to allow incorporation of the nucleic acid to be delivered to the arterial cell and its release when the hydrophilic polymer contacts the arterial cell. Preferably, the hydrophilic polymer is a hydrogel polymer. Other hydrophilic polymers will work, so long as they can retain the genetic material of the present invention, so that, on contact with arterial cells, transfer of genetic material occurs.

Suitable hydrogel polymers include, for example, those selected from the group consisting of polycarboxylic acids, cellulosic polymers, gelatin, polyvinylpyrrolidone, maleic anhydride polymers, polyamides, polyvinyl alcohols, and polyethylene oxides. The hydrogel polymer is preferably polyacrylic acid.

Without wishing to be bound by theory, one reason that the use of hydrogel, and particularly with hydrogel coated balloon catheters, is believed to provide improved results over, for example, prior treatments with double balloon catheters, is that the use of standard balloon catheters with hydrogel surfaces causes the hydrogel not only to contact the endothelial cells which line the interior of the arteries, but also displaces the endothelial cells sufficiently to permit contact between the hydrogel and the smooth muscle cells which underlie the endothelial cell layer. This permits expression of polypeptides in different arterial cell types, which enhances the kinds and amounts of therapeutic polypeptides which can be produced in accordance with this invention. For example, as indicated in the examples below, the present method successfully produces sufficient amounts of vascular endothelial growth factor (VEGF) to cause angiogenesis downstream from a DNA/arterial contact point, despite the fact that VEGF is not normally produced even by transformed endothelial cells, but is produced by smooth muscle cells of the type that surround the endothelial cells in the artery.

The arterial cell may be contacted with the hydrophilic polymer incorporating the DNA by means of an applicator such as a catheter which is coated with the DNA-bearing hydrophilic polymer. Preferably, the applicator can exert some pressure against the arterial cells, to improve contact between the nucleic acid-bearing hydrophilic polymer and the arterial cells. Thus a balloon catheter is preferred. Preferably, the hydrophilic polymer coats at least a portion of an inflatable balloon of the balloon catheter.

The present invention further includes compositions comprising hydrophilic polymers incorporating nucleic acid. Preferably the hydrophilic polymer is a hydrogel and the nucleic acid is DNA which encodes an angiogenic protein.

The present invention also provides kits for application of genetic material to the interior of an artery or similar bodily cavity, comprising a substrate, such as a catheter or a suitably shaped rod, and a source of genetic material comprising the DNA coding for the desired therapeutic polypeptide. Preferably, the present invention is directed to a catheter adapted for insertion into a blood vessel, having a balloon element adapted to be inserted into the vessel and expandable against the walls of the

vessel. At least a portion of the balloon element is defined by a coating of a hydrophilic polymer, and incorporated within the hydrophilic polymer coating, a nucleic acid to be delivered to the arterial cell. The hydrophilic polymer is preferably a hydrogel polymer, most preferably a hydrophilic polyacrylic acid polymer.

The present invention also provides a method for inducing angiogenesis in a desired target tissue, comprising delivering a DNA encoding an angiogenic protein to an arterial cell in an artery or blood vessel perfusing the target tissue.

Other aspects of the invention are discussed infra.

As used herein the term "angiogenic protein" means any protein, polypeptide, mutein or portion thereof that is capable of inducing the formation of new blood vessels. Such proteins include, for example, acidic and basic fibroblast growth factors (aFGF and bFGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor .alpha. and .beta. (TGF-.alpha. and TFG-.beta.), platelet-derived endothelial growth factor (PD-ECGF), platelet-derived growth factor (PDGF), tumor necrosis factor .alpha. (TNF-.alpha.), hepatocyte growth factor (HGF) and insulin like growth factor. Preferably, the angiogenic protein contains a secretory signal sequence allowing for secretion of the protein from the arterial cell. VEGF is a preferred protein.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1(a) and 1(b) show the rabbit ischemic hindlimb model. FIG. 1(a) is a representative angiogram recorded 10 days after surgery. Once the femoral artery is excised, thrombotic occlusion of the external lilac artery extends retrograde to its origin from the common lilac (arrow). Open arrow indicates the site of arterial gene transfer. In FIG. 1(b) the shaded segment of femoral artery has been excised.

FIGS. 2(a), 2(b) and 2(c) illustrate (a) RT-PCR analysis of transfected arteries, (b) Southern blot analysis of RT-PCR products and (c) nucleotide sequence of the RT-PCR product from transfected rabbit lilac artery. In FIGS. 2(a) and 2(b) the expression of the human VEGF mRNA was evident in the rabbit lilac artery (lane 4) and cultured rabbit vascular smooth muscle cells (lane 6, positive control) which were transfected with human VEGF gene. Arrows indicate position of VEGF band at 258 bp. Lane 1 depicts the results using a molecular weight marker, namely pGEM3zf(-) digested with Hae III; lane 2 is a negative control (no RNA); lane 3 is a second negative control (rabbit lilac artery transfected with .beta.-galactosidase expression plasmid); and lane 5 is a further negative control (PCR analysis of the VEGF-transfected lilac artery excluding the reverse transcriptase reaction). FIG. 2(c) shows the nucleotide sequence of the RT-PCR product from a transfected rabbit lilac artery. Direct sequencing of the 258 bp bands obtained by RT-PCR confirmed that this band represented the human VEGF sequence. The sequence designated in 2(c) corresponds to amino acids 69 to 75 of the VEGF peptide. Asterisks denote the nucleotides which are not conserved among different species of the VEGF gene (rat, mouse, bovine, guinea pig) demonstrating that the exogenous human gene was amplified by the RT-PCR procedure.

FIGS. 3A, 3B, 3C, 3D, 3E and 3F comprise internal lilac angiography of a control rabbit at (A) day 0 (pre-transfection), (B) day 10, and (C) day 30 post-transfection, and of a VEGF-transfected rabbit at (D) day 0, (E) day 10, and (F) day 30 post-transfection. In contrast to the control, angiographic examination of the VEGF-transfected animal discloses extensive collateral artery formation.

FIGS. 4(a), 4(b) and 4(c) are graphs illustrating the effect of VEGF-transfection on revascularization

in an ischemic limb model. FIG. 4(a) the angiographic score at day 0 (immediately prior to transfection), and days 10 and 30 post-transfection. FIG. 4(b) Calf Blood pressure ratio at day 0, and at days 10 and 30 post-transfection. FIG. 4(c) depicts capillary density at day 30 post-transfection. (*p<0.05,**p<0.01)

FIGS. 5(a) and 5(b) illustrate alkaline phosphatase staining of ischemic hindlimb muscle, counterstained with eosin. FIG. 5(a) depicts the muscle of an animal transfected with pGSVLacZ. FIG. 5(b) depicts the muscle of an animal transfected with phVEGF.sub.165. The dark staining indicates capillaries as shown by the arrows.

FIG. 6 illustrates a diagrammatical cross section of a balloon catheter having a hydrophilic surface bearing genetic material in accordance with the present invention, in place within an artery.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for the delivery of nucleic acid to an arterial cell comprising contacting the cell with a hydrophilic polymer incorporating the nucleic acid.

The nucleic acid may be any nucleic acid which when introduced to the arterial cells provides a therapeutic effect. The nucleic acid is selected based upon the desired therapeutic outcome. For example, in the treatment of ischemic diseases, one genetic material of choice would be a DNA encoding an angiogenic protein. DNA useful in the present invention include those that encode hormones, enzymes, receptors or drugs of interest. The DNA can include genes encoding polypeptides either absent, produced in diminished quantities, or produced in mutant form in individuals suffering from a genetic disease. Additionally it is of interest to use DNA encoding polypeptides for secretion from the target cell so as to provide for a systemic effect by the protein encoded by the DNA. Specific DNA's of interest include those encoding hemoglobin, interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-4, interleukin-5, interleukin-6, interleukin-7, interleukin-8, interleukin-9, interleukin-10, interleukin-11, etc., GM-CSF, G-CSF, M-CSR, human growth factor, insulin, factor VIII, factor IX, tPA, LDL receptors, tumor necrosis factor, PDGF, EGF, NGF, IL-1ra, EPO, .beta.-globin and the like, as well as biologically active muteins of these proteins. The nucleic acid utilized may also be "anti-sense" DNA or RNA, which binds to DNA or RNA and blocks the production of harmful molecules. In addition, the DNA carried to the arterial cells in accordance with the present invention may code for polypeptides which prevent the replication of harmful viruses or block the production of smooth muscle cells in arterial walls to prevent restenosis.

Antisense RNA molecules are known to be useful for regulating translation within the cell. Antisense RNA molecules can be produced from the corresponding gene sequences. The antisense molecules can be used as a therapeutic to regulate gene expression associated with a particular disease.

The antisense molecules are obtained from a nucleotide sequence by reversing the orientation of the coding region with regard to the promoter. Thus, the antisense RNA is complementary to the corresponding mRNA. For a review of antisense design see Green, et al., Ann. Rev. Biochem. 55:569-597 (1986), which is hereby incorporated by reference. The antisense sequences can contain modified sugar phosphate backbones to increase stability and make them less sensitive to RNase activity. Examples of the modifications are described by Rossi, et al., Pharmacol, Ther. 50(2):245-354, (1991).

In certain therapeutic applications, such as in the treatment of ischemic diseases, it may be desirable to induce angiogenesis, i.e., the formation of new blood vessels. For such applications, DNA's encoding growth factors, polypeptides or proteins, capable of inducing angiogenesis are selected. Folkman, et

al., Science, 235:442-447 (1987). These include, for example, acidic and basic fibroblast growth factors (aFGF and bFGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor .alpha. and .beta. (TGF-.beta. and TGF-.beta.), platelet-derived endothelial cell growth factor (PD-ECGF), platelet-derived growth factor (PDGF) itself, tumor necrosis factor .alpha. (TNF-.alpha.), hepatocyte growth factor (HGF) and insulin like growth factor. See, Klagsbrun, et al., Annu. Rev. Physiol., 53:217-239 (1991) and Folkman, et al., J. Biol. Chem. 267:10931-10934 (1992). Muteins or fragments of an angiogenic protein may be used as long as they induce or promote the formation of new blood vessels.

Recent investigations have established the feasibility of using recombinant formulations of such angiogenic growth factors to expedite and/or augment collateral artery development in animal models of myocardial and hindlimb ischemia. See, Baffour, et al., J. Vasc. Surg., 16:181-191 (1992) (bFGF); Pu, et al, Circulation, 88:208-215 (1993) (aFGF); Yanagisawa-Miwa, et al., Science, 257:1401-1403 (1992) (bFGF); Ferrara, et al., Biochem. Biophys. Res. Commun., 161:851-855 (1989) (VEGF).

VEGF was also purified independently as a tumor-secreted factor that included vascular permeability by the Miles assay (Keck, et al, Science, 246:1309-1342 (1989) and Connolly, et al., J. Biol Chem., 264:20017-20024 (1989)), and thus its alternate designation, vascular permeability factor (VPF). VEGF is a preferred angiogenic protein. Two features distinguish VEGF from other heparin-binding, angiogenic growth factors. First, the NH.sub.2 terminus of VEGF is preceded by a typical signal sequence; therefore, unlike bFGF, VEGF can be secreted by intact cells. Second, its high-affinity binding sites, shown to include the tyrosine kinase receptors Flt-1 and Flt-1/KDR are present on endothelial cells. Ferrara, et al., Biochem. Biophys. Res. Commun., 161:851-855 (1989) and Conn, et al., Proc. Natl. Acad. Sci. USA, 87:1323-1327 (1990). (Interaction of VEGF with lower affinity binding sites has been shown to induce mononuclear phagocyte chemotaxis). Shen, et al., Blood, 81:2767-2773 (1993) and Clauss, et al., J. Exp. Med., 172:1535-1545 (1990).

Evidence that VEGF stimulates angiogenesis in vivo had been developed in experiments performed on rat and rabbit cornea (Levy, et al., Growth Factors, 2:9-19 (1989) and Connolly, et al., J. Clin. Invest., 84:1470-1478 (1989)), the chorioallantoic membrane (Ferrara, et al., Biochem Biophys Res Commun., 161:851-855 (1989)), and the rabbit bone graft model. Connolly, et al., J. Clin. Invest., 84:1470-1478 (1989).

Preferably, the angiogenic protein contains a secretory signal sequence that facilitates secretion of the protein from the arterial cell. Angiogenic proteins having native signal sequences, e.g., VEGF, are preferred. Angiogenic proteins that do not have native signal sequences, e.g., bFGF, can be modified to contain such sequences using routine genetic manipulation techniques. See, Nabel et al., Nature 362:844 (1993).

The nucleotide sequence of numerous peptides and proteins, including angiogenic proteins, are readily available through a number of computer data bases, for example, GenBank, EMBL and Swiss-Prot. Using this information, a DNA segment encoding the desired may be chemically synthesized or, alternatively, such a DNA segment may be obtained using routine procedures in the art, e.g, PCR amplification.

To simplify the manipulation and handling of the DNA, prior to introduction to the arterial cell, the DNA is preferably inserted into a vector, e.g., a plasmid vector such as pUC118, pBR322, or other known plasmid vectors, that includes, for example, an E. Coli origin of replication. See, Sambrook, et al., Molecular Cloning:A Laboratory Manual, Cold Spring Harbor Laboratory press, (1989). The plasmid vector may also include a selectable marker such as the .beta.-lactamase gene for ampicillin

resistance, provided that the marker polypeptide does not adversely effect the metabolism of the organism being treated. Additionally, if necessary, the DNA may be operably linked to a promoter/enhancer region capable of driving expression of the protein in the arterial cell. An example of a suitable promoter is the 763-base-pair cytomegalovirus (CMV) promoter. Normally, an enhancer is not necessary when the CMV promoter is used. The RSV and MMT promoters may also be used. Certain proteins can expressed using their native promoter.

If desired, the DNA may be used with a microdelivery vehicle such as cationic liposomes and adenoviral vectors. For a review of the procedures for liposome preparation, targeting and delivery of contents, see Mannino and Gould-Fogerite, Bio Techniques, 6:682 (1988). See also, Felgner and Holm, Bethesda Res. Lab. Focus, 11(2):21 (1989) and Maurer, R. A., Bethesda Res. Lab. Focus, 11 (2):25 (1989). Replication-defective recombinant adenoviral vectors, can be produced in accordance with known techniques. See, Quantin, et al., Proc. Natl. Acad. Sci. USA, 89:2581-2584 (1992); Stratford-Perricadet, et al., J. Clin. Invest., 90:626-630 (1992); and Rosenfeld, et al., Cell, 68:143-155 (1992).

In certain situations, it may be desirable to use DNA's encoding two or more different proteins in order optimize the therapeutic outcome. For example, DNA encoding two angiogenic proteins, e.g., VEGF and bFGF, can be used, and provides an improvement over the use of bFGF alone. Or an angiogenic factor can be combined with other genes or their encoded gene products to enhance the activity of targeted cells, while simultaneously inducing angiogenesis, including, for example, nitric oxide synthase, L-argine, fibronectin, urokinase, plasminogen activator and heparin.

The hydrophilic polymer is selected to allow incorporation of the DNA to be delivered to the arterial cell and its release when the hydrophilic polymer contacts the arterial cell.

Preferably, the hydrophilic polymer is a hydrogel polymer, a cross-linked polymer material formed from the combination of a colloid and water. Cross-linking reduces solubility and produces a jelly-like polymer that is characterized by the ability to swell and absorb liquid, e.g., that containing the DNA. Suitable hydrogel polymers include, for example, those selected from the group consisting of polycarboxylic acids, cellulosic polymers, gelatin, polyvinylpyrrolidone, maleic anhydride polymers, polyamides, polyvinyl alcohols, and polyethylene oxides. Preferred hydrogels are polyacrylic acid polymers available as HYDROPLUS (Mansfield Boston Scientific Corp., Watertown, Mass.) and described in U.S. Pat. No. 5,091,205.

The nucleic acid in aqueous solution is incorporated into the hydrophilic polymer to form a nucleic acid-hydrophilic polymer composition. The nucleic acid is incorporated without complexing or chemical reaction with the hydrophilic polymer, and is preferably relatively freely released therefrom when placed in contact with the arterial cells. The resulting structure comprises a support, e.g. the balloon of the balloon catheter, on which is mounted the hydrogel, in or on which is incorporated the desired DNA and its associated vehicle, e.g., phage or plasmid vector. The hydrophilic polymer is preferably adhered to the support, so that after application of the DNA to the target cells, the hydrophilic polymer is removed with the support.

An arterial cell is contacted with the nucleic acid-hydrophilic polymer composition by any means familiar to the skilled artisan. The preferred means is a balloon catheter having the hydrophilic polymer on its outer surface, which permits the contact between the hydrophilic polymer bearing the nucleic acid to be transferred and the arterial cells to be made with some pressure, thus facilitating the transfer of the nucleic acid to the cells. However, other supports for the hydrophilic polymer are also useful, such as catheters or solid rods having a surface of hydrophilic polymer. Preferably, the catheters or

rods or other substrates which are flexible, to facilitate threading through the arteries to reach the point of intended application. For cells that are not in tubular arteries, other types of catheters, rods or needles may be used.

When a hydrophillic arterial balloon is used, it is not necessary to protect the balloon prior to inflation, since relatively little of the nucleic acid is lost in transit to the treatment site until the balloon is inflated and the hydrophilic polymer bearing the nucleic acid is pressed against the arterial cells. When hydrophilic polymer-surfaced catheters or rods are used as the vehicle or substrate, the surface can protected, e.g. by a sheath, until the point of intended application is reached, and then the protection removed to permit the hydrophilic polymer bearing the nucleic acid to contact the arterial cells.

The vehicle, be it arterial balloon, catheter, flexible rod or other shaped vehicle, can be furnished with means to assist in accurate placement within the intended body cavity. For example, it can be furnished with a radioactive element, or made radio-opaque, furnished with means permitting easy location using ultrasound, etc.

Preferably, the nucleic acid-hydrophilic composition contacts the arterial cell by means of a catheter. The catheter is preferably a balloon catheter constructed for insertion in a blood vessel and has a catheter shaft and an expandable dilation balloon mounted on the catheter shaft. At least a portion of the exterior surface of the expandable portion is defined by a coating of a tenaciously adhered hydrophilic. Incorporated in the hydrophilic polymer is an aqueous solution of the DNA to be delivered to the arterial cells.

In general, when dry, the hydrophilic polymer (preferably hydrogel) coating is preferably on the order of about 1 to 10 microns thick, with a 2 to 5 micron coating typical. Very thin hydrogel coatings, e.g., of about 0.2-0.3 microns (dry) and much thicker hydrogel coatings, e.g., more than 10 microns (dry), are also possible. Typically, hydrogel coating thickness may swell by about a factor of 2 to 10 or more when the hydrogel coating is hydrated.

Procedures for preparing a balloon with a hydrogel coating are set forth in U.S. Pat. No. 5,304,121, the disclosure of which is incorporated herein by reference.

A representative catheter is set forth in FIG. 6. Referring to FIG. 6, 1 is the wall of the blood vessel. The figure shows the catheter body 2 held in place by the inflation of an inflation balloon 3. The balloon comprises a hydrogel coating 4 incorporating DNA 5.

In use, the DNA, for example, is applied ex vivo to the hydrophilic polymer coating of the balloon. To facilitate application, the balloon may be inflated. If necessary, the polymer may be dried with warm air and the DNA application repeated. The amount of DNA to be applied to the arterial surface depends on the purpose of the DNA and the ability of the DNA to be expressed in the arterial cells. Generally, the amount of naked DNA applied to the balloon catheter is between about 0.1 and 100 .mu.g/mm.sup.2, more preferably between about 0.5 and about 20 .mu.g/mm.sup.2, most preferably between about 1.5 and about 8 .mu.g/mm.sup.2. Preferably, between 0.5 mg and 5 mg of DNA are applied to the hydrogel coating of a balloon catheter having an inflated lateral area of about 630 mm.sup.2 (e.g., a balloon catheter having an inflated diameter of about 5 mm and a length of about 40 mm), providing a surface having about 0.8 to about 8 .mu.g/mm.sup.2 of DNA when the balloon is inflated and contacts the interior of the artery. More preferably, between 1 mg and 3 mg of DNA are applied to the polymer, providing a DNA loading of about 1.6 to about 4.8 .mu.g/mm.sup.2.

The catheter is inserted using standard percutaneous application techniques and directed to the desired location, e.g., an artery perfusing the target tissue. For example, in the treatment of patients with occlusive peripheral arterial disease (PAD), the balloon is directed towards an artery of the leg, e.g., lilac. Once the balloon has reached its desired location, it is inflated such that the hydrogel coating of the balloon contacts the arterial cells located on the walls of the artery and remains inflated for a time sufficient to allow transfer of the DNA encoding the angiogenic protein from the hydrogel to the arterial cells. Preferred periods of balloon inflation range from 30 seconds to 30 minutes, more preferably 1 minute to 5 minutes. Surprisingly, that is normally sufficient time to permit transfer of the DNA by the method of the present invention.

Once transferred, the DNA coding for the desired therapeutic polypeptide is expressed by the arterial cells for a period of time sufficient for treatment of the condition of interest. Because the vectors containing the DNA of interest are not normally incorporated into the genome of the cells, however, expression of the protein of interest takes place for only a limited time. Typically, the therapeutic protein is only expressed in therapeutic levels for about two days to several weeks, preferably for about 1-2 weeks. Reapplication of the DNA can be utilized to provide additional periods of expression of the therapeutic polypeptide. If desired, use of a retrovirus vector to incorporate the heterologous DNA into the genome of the arterial cells will increase the length of time during which the therapeutic polypeptide is expressed, from several weeks to indefinitely.

In one preferred application, the DNA-hydrogel polymer composition can be used to deliver a DNA encoding an angiogenic protein to an arterial cell in an artery or blood vessel perfusing the target tissue. Expression of the angiogenic protein and its secretion from the arterial cell induces angiogenesis, i.e., the formation of new blood vessels, in target tissues perfused by the artery or blood vessels, allowing for the treatment of diseases associated with vascular occlusion such as limb ischemia, ischemic cardiomyopathy, myocardial ischemia, cerebral ischemia and portal hypertension.

The present invention makes genetic treatment possible which can correct heretofore incorrectable problems.

The present invention is further illustrated by the following examples. These examples are provided to aid in the understanding of the invention and are not construed as a limitation thereof.

EXAMPLE 1

Direct Gene Transfer with Hydrogel Polymer Balloon Catheter Applied to an Angioplasty Catheter Balloon Can be Used to Effect Direct Gene Transfer to the Arterial Wall.

DNA solution was applied to the surface of an angioplasty catheter balloon with a hydrogel polymer (marketed under the mark Slider.TM. with Hydroplus.RTM. by Mansfield Boston Scientific Corp., Watertown, Mass.). The catheter was constructed with a single polyethylene balloon, 2.0 mm in diameter and 2.0 cm in length. The Hydroplus.RTM. coating consists of a hydrophilic polyacrylic acid polymer, crosslinked via an isocyanate onto the balloon to form an ultra-high molecular weight hydrogel with tight adherence to the balloon surface. The thickness of the hydrogel coating when dry measures between 3-5 .mu.m; upon exposure to an aqueous environment, the coating swells to 2-3 times its initially dry thickness. In order to apply DNA to the catheter, the balloon was inflated to 4 atm, following which 20 .mu.l of DNA solution were pipetted and distributed onto the balloon surface using a sterile pipette tip. After the balloon's hydrogel polymer was covered with a homogeneous film of DNA solution, the hydrogel was dried with warm air. The above procedure was then repeated, resulting in a total of 40 .mu.l of DNA solution applied to the balloon.

For percutaneous application, luciferase DNA concentration was 3.27 .mu.g/.mu.l. DNA was dissolved in TE buffer (10 mM Tris, 1 mM EDTA).

(Attempts were made to apply DNA solution to standard uncoated balloons as well. The hydrophobic surface of the polyethylene balloon, however, made it impossible to cover the balloon with a film of DNA solution.)

To determine the total amount of DNA which is successfully absorbed onto the balloon surface, 5 hydrogel balloons were coated with 40 .mu.l DNA (2 .mu.g DNA/.mu.l) containing a small amount of .sup.35 S-labeled luciferase plasmid. (Levy, et al., Growth Factors, 2:1535-1545 (1990)). A random primed DNA labeling kit (United States Biochemical, Cleveland, Ohio) was used for the labeling reaction an unincorporated nucleotides were remove by ethanol precipitation. After the coating procedure, the catheter tip was placed in 0.5 ml water for 15 minutes at room temperature, and 1.0 ml gel solubilizer (Solvable,.TM. New England Nuclear, Boston, Mass.) for 3 hours at 50.degree. C. to dissolve the gel before the scintillation fluid was added. The amount of DNA on the balloon was calculated from the quotient: [counts per minute (cpm) in a scintillation vial containing the balloon]/[cpm in a vial containing 40 .mu.l of the same lot of labeled DNA (80 .mu.g)]. Scintillation counts were corrected for quench and chemiluminescence.

After coating hydrogel balloons with 40 .mu.l of DNA solution (containing 80 .mu.g of radiolabeled DNA), and drying the gel, the magnitude of DNA retained on the hydrogel balloon was determined by comparing the amount of radioactivity on the balloons to the amount of radioactivity in 40 .mu.l of the original radiolabeled DNA solution. Scintillation counting revealed that 97.+-.2% (n=5) of the radioactively labeled DNA remained on the hydrogel coated balloon, corresponding to 78.+-.1.5 .mu.g of luciferase DNA.

Reporter Genes

The firefly luciferase gene and the gene for nuclear-specific .beta.-galactosidase (.beta.-gal) were used as reporter genes to monitor the results of the transfection procedures. The luciferase expression vector, pRSVLUC (courtesy of Dr. Allen Brasier, Massachusetts General Hospital, Boston, Mass.), consist of a full length Photinus pyralis luciferase cDNA (pJD 204) (de Wet et al., 1987) inserted into a PGEM3-plasmid (Brasier et al. Biotechniques, 7:1116-1122 (1989)), under the control of Rous sarcoma virus long terminal repeat (RSV-LTR) promoter. The pGSVLacZ vector contains the simian virus (SV40) large tumor nuclear location signal fused to the lacZ gene (nls .beta.-gal) (Bonnerot et al., Proc. Natl. Acad. Sci. U.S.A., 84:6795-6799 (1987)) (gift from Dr. Claire Bonnerot, Institut Pasteur, Paris, France), inserted into a pGEM1-plasmid. Nuclear staining identifies the exogenous construct designed to permit nuclear translocation, and thus distinguishes expression of the transgene from endogenous (cytoplasmic) .beta.-gal activity. Previous concerns (Lim et al., Circulation, 83:2007-2011 (1991)) regarding nonspecificity of blue staining resulting from .beta.-gal are thus eliminated.

Analysis of Luciferase Activity

The magnitude of gene expression was determined by measuring luciferase activity as described previously (Leclerc et al., J. Clin. Invest., 90:936-944 (1992)) using the Luciferase Assay System (Promega, Madison, Wis.). Briefly, frozen arteries were homogenized and dissolved in 300 .mu.l of Cell Culture Lysis Reagent (Promega) containing 1 mg/ml bovine serum albumin. Three different 20- .mu.l aliquots prepared from each transfected specimen were mixed in a sample tube with 100 .mu.l of

Luciferase Assay Reagent (Promega, Madison, Wis.) and inserted into a luminometer (Model 20e, Turner Design, Sunnyvale, Calif.) that reports results on a scale established to yield as low as 10.sup.-3 Turner light units (TLU). The specimen's total luciferase activity was calculated from the mean of the three aliquots analyzed. The luciferase values were in the linear range of a standard curve derived from samples with a known amount of luciferase (Sigma, St. Louis, Mo., catalogue #L9009). The lyophilized luciferase was, according to the manufacturer's instructions, dissolved in sterile water and further diluted in Cell Culture Lysis Reagent with 1 mg/ml bovine serum albumin. The following equation was used to convert TLU into pg luciferase: Luciferase [pg]=-0.08+0.051 TLU. Using this formula, 100 TLU corresponds to 5.0 pg of luciferase. It must be noted that the specific activity of luciferase standards from different vendors can vary considerably (Wolff, et al., Biotechniques, 11:474-485 (1991)); therefore, direct comparisons of luciferase reported by different groups must be made with caution, especially when the origin of the standard used is not specified.

Percutaneous Transfection

Percutaneous gene transfer experiments with the luciferase gene were performed in 13 rabbits using a catheter with a balloon to which a 20 .mu.m hydrogel coating had been applied and which was advanced through a 5 F teflon sheath. The balloon was advanced beyond the distal tip of the sheath, coated with 130 .mu.g luciferase DNA, and pulled back into the sheath to protect the balloon from subsequent contact with blood. The sheath and the angioplasty catheter were then introduced via the right carotid artery and advanced to the left common lilac artery under fluoroscopic control. The balloon catheter was advanced 2 cm further (beyond the distal sheath tip) into the external lilac artery and inflated there for 1 or 5 min. Following balloon deflation, the catheter system was removed. In 10 animals, the transfected external lilac artery as well as the contralateral control artery were removed 3 days later, weighed, and assayed for luciferase activity. In 3 additional animals, which had been transfected for 5 min. only, the arteries were excised 14 days after gene transfer. In these 3 animals we also removed the left femoral artery to check for luciferase expression directly downstream of the transfected segment.

Results

Luciferase expression was detected in all 10 (100%) percutaneously transfected arteries excised after 3 days, whether inflated for 5 min (386.+-.299 TLU, n=5) or 1 min (113.+-.59 TLU, n=5).

Three additional animals, in which balloons were inflated for 5 min only, were sacrificed after 14 days. Individual luciferase expression was 152.6, and 16 TLU, respectively (mean=58.+-.47 TLU). In this series, we also measured luciferase in the adjacent femoral artery, which was not inflated. Luciferase expression in all these arteries was undistinguishable from background activity (mean 0.04.+-.0.29 TLU).

The findings demonstrate that endoluminal vascular gene transfer can be achieved successfully and consistently with pure DNA applied to a standard angioplasty catheter balloon coated with hydrogel polymer. The hydrogel provides the absorbable medium to which one may apply a solution of pure DNA. Drying of the gel results in a layer of concentrated DNA which is then transferred to the arterial wall as the balloon contacts the arterial wall coincident with balloon inflation. Experiments with radiolabeled DNA established that 97% of DNA applied in aqueous solution to the hydrogel-coated balloon was still present on the balloon after drying of the gel. Autoradiograms of the arterial wall demonstrated that inflation of the hydrogel balloon results in DNA uptake which is distributed across the full thickness of the arterial wall. DNA was shown to penetrate the intact internal elastic lamina and was distributed intracellularly as well as extracellularly.

Despite elimination of accessory transfection vehicles in this example, both the frequency of successful transfection and the magnitude of reporter gene expression achieved were superior to that previously reported from our laboratory (Leclerc, et al., J. Clin Invest., 90:936-944 (1992)) and comparable to the results achieved by others (Chapman, et al., Circ. Res., 71:27-33 (1992) and Lim, et al., Circulation, 83:2007-2011 (1991)) using alternative delivery schemes. The success rate of transfection in our rabbit model as measured by expression of the luciferase transgene was 100% (37 of 37 artery segments), even in those cases in which the inflation time was reduced to one minute. The duration of inflation within a range from 10 to 30 minutes did not have significant impact on transfection efficiency, a feature which would be expected to facilitate human arterial, particularly coronary, gene transfer.

EXAMPLE 2

Induction of Angiogeneses In Vivo

Methods

Animal Model (FIG. 1).

The angiogenic response to transfection of the gene for vascular endothelial growth factor (VEGF) was investigated using a rabbit ischemic hindlimb model. Takeshita, et al., J. Clin. Invest., 93:662-670 (1994) and Pu, et al., J. Invest Surg., (In Press). All protocols were approved by St. Elizabeth's Institutional Animal Care and Use Committee. Male New Zealand White rabbits weighing 4-4.5 kg (Pine Acre Rabbitry, Norton, Mass.) were anesthetized with a mixture of ketamine (50 mg/kg) and acepromazine (0.8 mg/kg) following premedication with xyazine (2.5 mg/kg). A longitudinal incision was then performed, extending inferiorly from the inguinal ligament to a point just proximal to the patella. The limb in which the incision was performed--right versus left--was determined at random at the time of surgery by the surgeon. Through this incision, using surgical loops, the femoral artery was dissected free along its entire length; all branches of the femoral artery, including the inferior epigastric, deep femoral, lateral circumflex and superficial epigastric arteries, were also dissected free. After further dissecting the popliteal and saphenous arteries distally, the external lilac artery as well as all of the above arteries were ligated. Finally, the femoral artery was completely excised from its proximal origin as a branch of the external lilac artery, to the point distally where it bifurcates into the saphenous and popliteal arteries. Once the femoral artery is excised, thrombotic occlusion of the external lilac artery extends retrograde to its origin from the common lilac (FIG. 1(a), arrow). As a result, the blood supply to the distal limb is dependent on the collateral arteries which may originate from the internal lilac artery. Accordingly, direct arterial gene transfer of VEGF was performed in to the internal lilac artery of the ischemic limb. Post-operatively, all animals were closely monitored. Analgesia (levorphanol tartrate 60 mg/kg, Roche Laboratories, Nutley, N.J.) was administered subcutaneously as required for evidence of discomfort throughout the duration of the experiment. Prophylactic antibiotics (enrofloxacin 2.5 mg/kg, Miles, Shawnee Mission, Kans.) was also administered subcutaneously for a total of 5 days post-operatively.

Plasmids and Smooth Muscle Cell (SMC) Transfection in Vitro.

Complementary DNA clones for recombinant human VEGF.sub.165, isolated from cDNA libraries prepared from HL60 leukemia cells, were assembled into a mammalian expression vector containing the cytomegalovirus promoter. Leung, et al., Science, 246:1306-1309 (1989). The biological activity of VEGF.sub.165 secreted from cells transfected with this construct (phVEGF.sub.165) was

previously confirmed by the evidence that media conditioned by transfected human 293 cells promoted the proliferation of capillary cells. Leung, et al., Science, 246:1306-1309 (1989).

To evaluate expression of phVEGF.sub.165 in vascular cells, rabbit arterial smooth muscle cells (SMCs) were transfected in vitro. Cells were cultured by explant outgrowth from the thoracic aorta of New Zealand White rabbits. The identity of vascular SMCs was confirmed morphologically using phase contrast microscopy and by positive immunostaining using a monoclonal antibody to smooth muscle .alpha.-actin (Clone 1A4, Sigma, St. Louis, Mo.). Cells were grown in the media (M199, GIBCO BRL, Gaithersberg, Md.) supplemented with 10% fetal bovine serum (FBD, GIBCO BRL). In vitro transfection was performed by incubating SMCs (1.48.times.10.sup.6 cells/10 cm plate) with 11.5 .mu.g of the plasmid DNA and 70 .mu.g of liposomes (Transfection-reagent, Boehringer Mannheim, Indianapolis, Ind.) as previously described. Pickering, et al., Circulation, 89:13-21 (1994). After completion of transfection, media was changed to 10% FBS. Culture supernatant was sampled at 3 days pot-transfection, and was analyzed by ELISA assay for VEGF protein. Houck, et al., J. Biol. Chem. 267:26031-26037 (1992).

The plasmid pGSVLacZ (courtesy of Dr. Claire Bonnerot) containing a nuclear targeted .beta.-galactosidase sequence coupled to the simian virus 40 early promoter (Bonnerot, et al., Proc. Natl. Acad. Sci. USA, 84:6795-6799 (1987)) was used for all the control transfection experiments.

Percutaneous Arterial Gene Transfer in Vitro.

An interval of 10 days between the time of surgery and gene transfer was allowed for post-operative recovery of rabbits and development of endogenous collateral vessels. Beyond this time-point, studies performed up to 90 days post-operatively (Pu, et al., J. Invest. Surg., (In Press)) have demonstrated no significant collateral vessel augmentation. At 10 days post-operatively (day 0), after performing a baseline angiogram (see below), the internal lilac artery of the ischemic limb of 8 animals was transferred with phVEGF.sub.165 percutaneously using a 2.0 mm hydrogel-coated balloon catheter (Slider.TM. with HYDROPLUS,.RTM. Boston Scientific, Watertown, Mass.). The angioplasty balloon was prepared (ex vivo) by first advancing the deflated balloon through a 5 Fr. teflon sheath (Boston Scientific), applying 400 .mu.g of phVEGF.sub.165 to the 20 .mu.m-thick later of hydrogel on the external surface of the inflated balloon, and then retracting the inflated balloon back into the protective sheath. The Sheath and angioplasty catheter were then introduced via the right carotid artery, and advanced to the lower abdominal aorta using a 0.014 inch guidewire (Hi-Torque Floppy II, Advanced Cardiovascular Systems, Temecula, Calif.) under fluoroscopic guidance. The balloon catheter was then advanced out of the sheath into the internal lilac artery of the ischemic limb, inflated for 1 min at 6 atmospheres, deflated, and withdrawn (FIG. 1(a), open arrow). An identical protocol was employed to transfect the internal iliac artery of 9 control animals with the plasmid pGSVLacZ containing a nuclear targeted .beta.-galactosidase sequence. Heparin was not administered at the time of transfection or angiography.

Evaluation of Angiogenesis in the Ischemic Limb.

Development of collateral vessels in the ischemic limb was serially evaluated by calf blood pressure measurement and internal lilac arteriography immediately prior to transfection (day 0), and then in serial fashion at days 10 and 30 post-transfection. On each occasion, it was necessary to lightly anesthetize the animal with a mixture of Ketamine (10 mg/kg) and acepromazine (0.16 mg/kg) following premedication with xyazine (2.5 mg/kg). Following the final 30-day follow-up, the animal was sacrificed, and tissue sections were prepared from the hindlimb muscles in order to perform analysis of capillary density. These analyses are discussed in detail below.

Calf Blood Pressure Ratio.

Calf blood pressure was measured in both hindlimbs using a Doppler Flowmeter (Model 1050, Parks Medical Electronics, Aloha, Oreg.), immediately prior to transfection (day 0), as well as on days 10 and 30. On each occasion, the hindlimbs were shaved and cleaned; the pulse of the posterior tibial artery was identified using a Doppler probe; and the systolic pressure of both limbs was determined using standard techniques. Takeshita, et al., J. Clin. Invest., 93:662-670 (1994). The calf blood pressure ratio was defined for each rabbit as the ratio of systolic pressure of the ischemic limb to systolic pressure of the normal limb.

Selective Internal Iliac Arteriography.

Collateral artery development in this ischemic hindlimb model originates from the internal lilac artery. Accordingly, selective internal lilac arteriography was performed on day 0 (immediately prior to transfection), and again on days 10 and 30 post-transfection as previously described. Takeshita, et al., J. Clin. Invest., 93:662-670 (1994). A 3 Fr. end-hole infusion catheter (Tracker-18, Target Therapeutics, San Jose, Calif.) was introduced into the right common carotid artery through a small cutdown, and advanced to the internal lilac artery at the level of the interspace between the seventh lumbar and the first sacral vertebrae. Following intra-arterial injection of nitroglycerin (0.25 mg, SoloPak Laboratories, Franklin Park, Ill.), a total of 5 ml of contrast media (Isovue-370, Squibb Diagnostics, New Brunswick, N.J.) was then injected using an automated angiographic injector (Medrad, Pittsburgh, Pa.) programmed to reproducibly deliver a flow rate of 1 ml per sec. Serial images of the ischemic hindlimb were then recorded on 105-mm spot film at a rate of 1 film per sec for at least 10 sec. Following completion of arteriography, the catheter was removed and the wound was closed. All of the above-described procedures were completed without the use of heparin.

Morphometric angiographic analysis of collateral vessel development was performed as previously described. Takeshita, et al., J. Clin. Invest., 93:662-670 (1994). A composite of 5-mm.sup.2 grids was placed over the medical thigh area of the 4-sec angiogram. The total number of grid intersections in the medical thigh area, as well as the total number of intersections crossed by a contrast-opacified artery were counted individually by a single observer blinded to the treatment regimen. An angiographic score was calculated for each film as the ratio of grid intersections in the medial thigh.

Capillary Density and Capillary/Myocyte Ratio.

The effect of VEGF gene transfer upon anatomic evidence of collateral artery formation was further examined by measuring the number of capillaries in light microscopic sections taken from the ischemic hindlimbs. Takeshita, et al., J. Clin. Invest., 93:662-670 (1994). Tissue specimens were obtained as transverse sections from the ischemic limb muscles at the time of sacrifice (day 30 post-transfection). Muscle samples were embedded in O.C.T. compound, (Miles, Elkhart, Ind.) and snap-frozen in liquid nitrogen. Multiple frozen sections (5 .mu.m in thickness) were then cut from each specimen on a cryostat (Miles), so that the muscle fibers were oriented in a transverse fashion, and two sections then placed on glass slides. Tissue sections were stained for alkaline phosphate using an indoxyl-tetrazolium method to detect capillary endothelial cells (Ziada, et al., Cardiovasc. Res., 18:724-732 (1984)), and were then counterstained with eosin. Capillaries were counted under a 20x objective to determine the capillary density (mean number of capillaries per mm.sup.2). A total of 20 different fields was randomly selected, and the number of capillaries counted. To ensure that analysis of capillary density was not overestimated due to muscle atrophy, or underestimated due to interstitial edema, capillaries identified at necropsy were also evaluated as a function of myocytes in the

histologic section. The counting scheme used to compute the capillary/myocyte ratio was otherwise identical to that used to compute capillary density.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR), Southern Blot Analysis, and Sequencing of RT-PCR Product.

The presence of human VEGF mRNA was detecting using RT-PCR. Arterial samples were obtained at 5 days post-transfection, and total cellular RNA was isolated using TRI REAGENT (Molecular Research Center, Cincinnati, Ohio) according to the manufacturer's instructions. Extracted RNA was treated with DNase I (0.5 .mu.l, 10 U/.mu.l, RNase-free, Message Clean kit, GenHunter, Boston, Mass.) at 37.degree. C. for 30 min to eliminate DNA contamination. The yield of extracted RNA was determined spectrophotometrically by ultraviolet absorbance at 260 nm. To check that the RNA was not degraded and electrophoresed through a 1% non-denaturing miniagarose gel. 0.5 .mu.g of each RNA sample was used to make cDNA in a reaction volume of 20 .mu.l containing 0.5 mM of each deoxynucleotide triphosphate (Pharmacia, Piscataway, N.J.), 10 mM dithiothreitol, 10 units of RNasin (Promega, Madison, Wis.), 50 mM Tris-HCI (pH 8.3), 75 mM KCL, 3 mM MgCl.sub.2, 1 .mu.g random hexanucleotide primers (Promega), and 200 units of M-MLV reverse transcriptase (GIBCO BRL). For greater accuracy and reproducibility, master mixes for a number of reactions were made up and aliquoted to tubes containing RNA. Reactions were incubated at 42.degree. C. for 1 hr, then at 95.degree. C. for 5 min to terminate the reaction. Twenty .mu.l of diethyl pyrocarbonate (DEPC) water was then added and 5 .mu.l of the diluted reaction (1/8th) was used on the PCR analysis. The optimized reaction in a total volume of 20 .mu.l contained 0.2 mM of each deoxynucleotide triphosphate, 3 mM MgCl.sub.2, 2 .mu.l PCR II buffer (Perkin-Elmer, Norwalk, Conn.; final concentrations, 50 mM KCL, 10 mM Tris-HCL), 5 ng/.mu.l (13.77 pmoles) of each primer, and 0.5 units of AmpliTaq DNA polymerase (Perkin-Elmer). The PCR was performed on a 9600 PCR system (Perkin-Elmer) using microamp 0.2. ml thin-walled tubes. Amplification was for 40-45 cycles of 94.degree. C. for 20 sec, 55.degree. C. for 20 sec, and 72.degree. C. for 20 sec, ending with 5 min at 72.degree. C. To test for false positives, controls were included with no RNA and no reverse transcriptase. A pair of oligonucleotide primers (22 mers) was designed to amplify a 258 bp sequence from the mRNA of human VEGF. To ensure specificity and avoid amplification of endogenous rabbit VEGF, each primer was selected from a region which is not conserved among different species. Sequences of primers used were: 5'-GAGGGCAGAATCATCACGAAGT-3' (sense) SEQ. ID NO:1 ; 5'-TCCTATGTGCTGGCCTTGGTGA-3' (antisense) SEQ. ID NO:2. RT-PCR products were transferred from agarose gels to nylon membranes (Hybond, Amersham, Arlington Heights, Ill.). The probe was 5' end-labelled with T4 polynucleotide kinase and [.beta.-.sup.32 P]ATP (Sambrook, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, (1989)) and hybridized to the nylon filters using Rapid Hybridization buffer (Amersham) according to manufacturer's instructions. To visualize hybridized bands, filters were exposed to X-ray film (Kodak Xar-5).

To confirm the identity of VEGF PCR products. DNA bands were excised from agarose gels, purified using Geneclean (BIO 101, La Jolla, Calif.), and sequenced directly (i.e. without subcloning) using dsDNA Cycle Sequencing System (GIBCO BRL) following the directions of manufacturer. The two VEGF primers used for PCR were 5' end-labeled with [.beta.-.sup.32 P]ATP and T.sub.4 polynucleotide kinase and used as sequencing primers to determine the sequence of both strands of the PCR product.

.beta.-Galactosidase Staining of Transfected lilac Arteries.

To evaluate the efficiency of in vivo arterial gene transfer, .beta.-galactosidase activity was determined

by incubation of arterial segments with 5-bromo-4-chloro-3-indolyl .beta.-D-galactosidase chromogen (X-Gall), Sigma) as previously described. Riessen, et al., Hum. Gene Ther., 4:749-758 (1993). Following staining with X-Gal solution, tissues were paraffin-embedded, sectioned, and counterstained with nuclear fast red. Nuclear localized .beta.-galactosidase expression of the plasmid pGSVLacZ cannot result from endogenous .beta.-galactosidase activity; accordingly, histochemical identification of .beta.-galactosidase within the cell nucleus was interpreted as evidence for successful gene transfer and gene expression. Cytoplasmic or other staining was considered non-specific for the purpose of the present study.

Statistics.

Results were expressed as means.+-.standard deviation (SD). Statistical significance was evaluated using unpaired Student's t test for more than two means. A value of $p < 0.05$ was interpreted to denote statistical significance.

Results

ELISA Assay for VEGF. To test the expression of the plasmid phVEGF.sub.165 in vascular cells, culture supernatant of VEGF-transfected SMCs (1.48.times.10.sup.6 cells/10 cm plate) was sampled at 3 days post-transfection, and analyzed by ELSA for VEGF protein. The media of VEGF-transfected SMCs contained an average of 1.5 .mu.g of VEGF protein (n=3). In contrast, culture media of .beta.-galactosidase-transfected SMCs (n=3) or non-transfected SMCs (n=3) did not contain detectable levels of VEGF protein.

RT-PCR, Southern Blot Analysis, and Sequencing of RT-PCR Product.

To confirm expression of human VEGF gene in transfected rabbit lilac arteries in vivo, we analyzed transfected arteries for the presence of human VEGF mRNA by RT-PCR. As indicated above, to ensure the specificity of RT-PCR for human VEGF mRNA resulting from successful transfection (versus endogenous rabbit VEGF mRNA), primers employed were selected from a region which is not conserved among different species. Arteries were harvested at 5 days post-transfection. The presence of human VEGF mRNA was readily detected in rabbit SMC culture (n=3) and rabbit lilac arteries (n=3) transfected with phVEGF.sub.165. Rabbit lilac arteries transfected with pGSVLacZ (n=3) were negative for human VEGF mRNA (FIG. 2(a)). Southern blot analysis was used to further confirm that the 158 bp bands obtained by RT-PCR did in fact correspond to the region between the two primers (FIG. 2(b)). Direct sequencing of the RT-PCR product document that this band represented the human VEGF sequence (FIG. 2(c)).

Angiographic Assessment.

The development of collateral vessels in the 5 rabbits transfected with phVEGF.sub.165 and 6 rabbits transfected with pGSVLacZ was evaluated by selective internal lilac angiography. FIG. 3 illustrates representative internal lilac angiogram recorded from both control and VEGF-transfected animals. In control animals, collateral artery development in the medial thigh typically appeared unchanged or progressed only slightly in serial angiogram recorded at days 0, 10, and 30 (FIGS. 3(a-c)). In contrast, in the VEGF-transfected group, marked progression of collateral artery was observed between days 10 and 30 (FIGS. 3, (d-f)). Morphometric analysis of collateral vessel development in the media thigh was performed by calculating the angiographic score as described above. At baseline (day 0), there was no significant difference in angiographic score between the VEGF-transfected and control groups (day 0:0.17.+-.0.02 vs 0.20.+-.0.06, p=ns). By day 30, however, the angiographic score in VEGF-

transfected group was significantly higher that in control group (0.47.+-.0.09 vs 0.34.+-.0.10, p<0.05) (FIG. 4(a)).

Calf Blood Pressure Ratio (FIG. 4(b)).

Reduction of the hemodynamic deficit in the ischemic limb following VEGF-transfection was confirmed by measurement of calf blood pressure ratio (ischemic/normal limb). The calf blood pressure ratio was virtually identical in both groups prior to transfection (0.23.+-.0.12 in VEGF-transfected animals, p=ns). By day 10 post-transfection, the blood pressure ration for VEGF-transfected rabbits was significantly higher than for the control rabbits (0.60.+-.0.12 vs 0.32.+-.0.14, p<0.01). At day 30, the blood pressure ratio for the VEGF-transfected group continued to exceed that of controls (0.70.+-.0.08 vs 0.50.+-.0.18, p<0.05).

Capillary Density and Capillary/Myocyte Ratio (FIGS. 4(c), 5).

A favorable effect of VEGF-transfection upon revascularization was also apparent at the capillary level. The medial thigh muscles of the ischemic limbs were histologically examined at day 30 post-transfection. Analysis of capillary density disclosed a value of 233.0.+-.60.9/mm.sup.2 in VEGF-transfected group versus 168.7.+-.31.5/mm.sup.2 in the control group (p<0.05). Analysis of capillary/myocyte ratio disclosed a value of 0.67.+-.0.15 in the VEGF-transfected group versus 0.48.+-.0.10 in the control group (p<0.05).

.beta.-Galactosidase Staining of Transfected Iliac Arteries.

To evaluate the efficiency of in vivo arterial gene transfer, transfected lilac arteries were harvested at 5 days post-transfection, and were used for .beta.-galactosidase histochemical analysis. In arteries transfected with nuclear targeted .beta.-galactosidase, evidence of successful transfection, indicated by dark blue nuclear staining, was observed in only<0.5% of total arterial cells. Arteries transfected with phVEGF.sub.165 were negative for nuclear staining.

EXAMPLE 3

Comparison of Double-Balloon Catheter Technique and Hydrogel-Coated Balloon Catheter Technique

Methods

Recombinant Adenoviral Vectors

Replication-defective recombinant adenoviral vectors, based on human adenovirus 5 serotype, were produced as previously described. Quantin, et al., Proc. Nat. Acad. Sci. USA, 89:2581-2584 (1992); Stratford-Perricaudet, et al., J. Clin. Invest., 90:626-630 (1992); and Rosenfeld, et al., Cell, 68:143-155 (1992). Ad-RSV.beta.gal contains the Escherichia coil lac Z gene and the SV40 early region nuclear localization sequence (nls). The nlslacZ gene encodes a nuclear-targeted .beta.-galactosidase under the control of the Rous sarcoma virus promoter. Ad-RSVmDys, used as a negative control, contains a human "minidystrophin" cDNA under the control of the same promoter. Ragot, et al., Nature, 361:647-650 (1993).

In Vivo Percutaneous Gene Transfer Procedures

All animal procedures were approved by the Institutional Animal Care and Use Committees of Faculte Bichat and St. Elizabeth's Hospital. Gene transfer was performed in the external lilac artery of 29 New Zealand white rabbits under general anesthesia and sterile conditions. Anesthesia was induced with intramuscular acepromazine and maintained with intravenous pentobarbital. Adenoviral stocks were used within 30 minutes of thawing.

1. Double-balloon catheter technique.

In 15 animals, Ad-RSV.beta.gal (2.10.sup.9 to 2.10.sup.10 plaque forming unites {pfu} in 2 ml PBS) was transferred to the right lilac artery, either normal (n=9) or previously denuded (n=6), using a 4 French double-balloon catheter (Mansfield Medical, Boston Scientific Corp., Watertown, Mass.) as previously described. Nabel, et al., Science, 244:1342-1344 (1989). The catheter was positioned in a segment of the artery which lacked angiographically visible side branches. The viral solution was maintained in contact with the arterial wall for 30 min. The left lilac artery of the same 15 animals was used as a control: in 7 animals no catheter was inserted, in 6 animals the endothelium was removed using balloon abrasion, and, in the 2 other animals, a double-balloon catheter was used in infuse Ad-RSVmDys (2.10.sup.9 pfu in 2 ml PBS).

2. Hydrogel-Coated Balloon Catheter Technique.

In 14 animals, a hydrogel-coated balloon catheter was used (Slider.TM. with Hydroplus.RTM., Mansfield Medical, Boston Scientific Corp., Watertown, Mass.). The balloon diameter (either 2.5 or 3.0 mm), was chosen to approximate a 1.0 balloon/artery ratio based on caliper measurement of magnified angiographic frames. Ad-RSV.beta.gal (1-2.10.sup.10 pfu in 100 .mu.l PBS) was applied to the polymer-coated balloon using a pipette as described above. The catheter was introduced into the right femoral artery through a protective sheath, the balloon was inflated at 1 atm, and the assembly was then advanced over a 0.014" guide wire to the external lilac artery where, after balloon deflation, the catheter alone was advanced 2 cm further and the balloon inflated for 30 minutes at 6 atm (ensuring nominal size of the inflated balloon). The contralateral lilac artery was in each case used as a control: in 9 animals no catheter or virus was introduced, in 2 the endothelium was removed, while in 3 a hydrogel-coated balloon catheter was used to transfer Ad-RSVmDys.

Detection of lacZ Expression in the Arterial Wall.

Three to seven days after transfection, the animals were sacrificed by pentobarbital overdose. To assess nlslacZ gene expression, the arteries were harvested and stained with X-Gal reagent (Sigma) for 6 hours, at 32.degree. C., as previously described. Sanes, et al., EMBO J., 5:3133-3142 (1986). Samples were then either mounted in OCT compound (Miles Laboratories Inc., Ill.) for cryosectioning or embedded in paraffin, cut into 6-.mu.m sections, and counterstained with hematoxylin and eosin or elastic trichrome. Expression of nlslacZ gene was considered positive only when dark blue staining of the nucleus was observed. To determine which cell types within the arterial wall expressed the transgene, immunohistochemical staining of X-Gal-stained arterial sections was performed, using a mouse monoclonal anti-.alpha.-actin primary antibody specific for vascular smooth muscle (HHF-35, Enzo Diagnostics, Farmingdale, N.Y.), and then a polyclonal peroxidase-labeled anti-mouse immunoglobulin G secondary antibody (Signet Laboratories, Dedham, Mass.).

Morphometric Analysis of nlslacZ Gene Expression in the Media.

For each transfected iliac artery, at least 2 samples were taken from the target-zone, and from each sample, at least 3 sections were examined by light microscopy after X-gal staining. Due to the

heterogeneity of .beta.-galactosidase activity on gross examination, the percentage of transfected medial cells per artery section was determined in regions showing high .beta.-galactosidase activity by counting stained versus total nuclei. The total numbers of studied medial cells were 14.10.sup.3 (n=50 sections) in the double-balloon catheter and the hydrogel-coated balloon catheter groups respectively.

Detection of Remote.sup.nlslacZ Gene Transfer and Expression.

Tissue samples from liver, brain, testes, heart, lungs, kidneys, contralateral limb skeletal muscle, and arterial segments adjacent to the treated arterial site were harvested immediately after sacrifice. For each specimen, nlslacZ gene presence and expression were assessed by polymerase chain reaction (PCR) and histochemistry (X-gal staining) respectively.

For PCR, genomic DNA was extracted from tissues by standard techniques. DNA amplification was carried out using oligodeoxynucleotide primers designed to selectively amplify Ad-RSV.beta.gal DNA over endogenous .beta.-galactosidase gene by placing one primer in the adenovirus sequence coding for protein 9 and the other primer in the lacZ sequence (5'-AGCCCGTCAGTATCGGCGGAATTC-3' (SEQ ID NO:3) and 5'-CAGCTCCTCGGTCACATCCAG-3' (SEQ ID NO:4) respectively, Genset, Paris, France). The reactions were performed in a DNA thermocycler (GeneAmp PCR System 9600, Perkin Elmer Cetus, Norwalk, Conn.) following 2 different protocols: a hold at 95.degree. C. for 3 min, 35 or 45 cycles of 95.degree. C. for 30 s, 65.degree. C. for 40 s, and 72.degree. C. for 1 min, then a final extension at 72.degree. C. for 5 min. When PCR was performed on plasmid DNA containing the nlslacZ gene used for the preparation of the adenoviral vector, or on positive liver samples obtained by deliberate systemic injection of Ad-RSV.beta.gal, the amplification reaction produced a 700 bp DNA fragment. To determine sensitivity of these procedures, DNA was extracted from liver of uninfected rabbits, aliquoted into several tubes, and spiked with dilutions of the plasmid containing the nlslacZ gene and used as a positive control. Following the amplification protocols described above, it was determined that the 35- or 45-cycle PCR could detect one copy of the nlslacZ gene in 3.102 and 3.104 cells respectively. DNA extractions and amplifications were performed simultaneously and in duplicate for studied tissues and positive controls.

Each tissue sample was also processed for histochemical analysis following the same protocol described for the arteries. For each specimen, at least 3 different segments were obtained, embedded in paraffin, and cut into at least 5 sections. Sections were counterstained with hematoxylin and eosin, and examined by light microscopy for the presence of deep blue nuclei indicative of .beta.-galactosidase expression. The number of positive cells as well as the total number of cells were counted. The total number of cells examined per sample ranged from 25.10.sup.3 to 115.10.sup.3.

Statistics

Results are expressed as mean.+-.standard deviation (SD). Comparisons between groups were performed using Student's t test for unpaired observations. A value of p<0.05 was accepted to denote statistical significance.

Results

Histological and Histochemical Analyses of Transfected Arteries Following Double-Balloon Catheter Delivery

Gross examination of all the arteries (n=15) following X-gal staining showed punctiform, heterogeneous, blue staining on the luminal aspect of the arteries, always limited to the area between

the two balloons. For the 9 normal arteries, microscopic examination disclosed dark blue nuclear staining, confined entirely to the endothelium. In contrast, when endothelial abrasion preceded transfection (n=6), X-gal staining imparted a mottled appearance to the luminal aspect of the artery. In these cases, microscopic examination showed that the endothelium had been removed and that the site of X-gal staining was subjacent to the intact internal elastic lamina, involving sparse medial cells. The identity of the transfected medial cells as smooth muscle cells was confirmed by immunohistochemical staining with monoclonal anti-.alpha.-actin antibody. Control arteries showed no nuclear blue staining.

Histological and Histochemical Analysis of Transfected Arteries Following Hydrogel-Coated Balloon Catheter Delivery

Gross examination of all the arteries after X-gal staining (n=14) showed dark blue, heterogeneous staining of the transfected site with a sharp boundary between the transfected segment and the bordering proximal and distal segments. Microscopic examination showed no residual intact endothelium; the continuity of the internal elastic lamina, in contrast, appeared preserved without apparent disruption. In the areas showing evidence of .beta.-galactosidase activity on gross examination, light microscopic examination revealed nearly continuous layers of cells with dark blue nuclear staining, generally limited to the superficial layers of the media; occasionally, sparsely distributed cells from deeper layers of the media expressed the transgene as well. Staining with monoclonal anti-.alpha.-actin antibody confirmed that transfected cells were vascular smooth muscle cells. No evidence of nuclear .beta.-galactosidase activity was seen in control arteries.

Morphometric Analysis of nlslacZ Gene Expression in the Media.

The percentage of transduced cells per artery section in regions showing high .beta.-galactosidase activity was significantly higher in the hydrogel-coated balloon catheter group than in the double-balloon catheter group (6.1.+-.2.3% vs. 0.4.+-.0.6%, p<0.0001).

Detection of Remote lacZ Gene Transfer and Expression in Other Organs

In all animals of both groups, gross and microscopic examination of X-gal stained tissue samples from liver, brain, testes, heart, lungs, kidneys, contralateral limb skeletal muscle, and arterial segments adjacent to the treated arterial site failed to show expression of nuclear-targeted .beta.-galactosidase, except in the liver of one rabbit in the double-balloon catheter group which disclosed a limited area of nuclear and peri-nuclear blue staining. In this area, less than 1/2.10.sup.3 cells expressed .beta.-galactosidase. In a few macrophages limited to samples removed from the lungs and testes of one hydrogel-coated balloon catheter treated rabbit, blue staining of the cytoplasm without nuclear staining was observed; the exclusively cytoplasmic location of .beta.-galactosidase activity in these cases, however, suggested that staining resulted from endogenous .beta.-galactosidase.

All of the above tissue samples were then screened by PCR. When the PCR was run for 35 cycles, the presence of DNA sequence specific for Ad-RSV.beta.gal was non-detectable, including in tissue samples from those animals with the highest percentage of transfected lilac arterial cells. Using an optimized protocol of 45 cycles, however, PCR was positive in the single liver that was observed to express .beta.-galactosidase, but in none of the other tissues.

This invention has been described in detail including the preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements thereon without departing from the spirit and scope of the invention as set forth in the claims.

HARVARD UNIVERSITY Gazette

The following articles appeared in the May 14, 1998, issue. Brief items have been omitted.

College Admission Yield Is Nearly 80%

Women's Studies in Religion Brings New Voices, Perspectives

Bone Drug Lowers Risk of Heart Disease

Virtual Press Room Open for Harvard Conference on Internet & Society

Notes

Labor Economist Myra Strober to Deliver Feminist Economics Lecture at Radcliffe Institute

Police Blotter

A Life of Service

NewsMakers

Peiser Appointed as Professor at Graduate School of Design

Study Finds that Governmental Procedure To Reduce Litigation Actually Leads to More Lawsuits

Knowles Elected Trustee Of Howard Hughes Medical Institute

Faculty To Meet with South Africa's Desmond Tutu, Truth Commission

'Radrugby': Bruised, Battered, Unbowed

Fragments of a Forgotten Past

FAS Administrative, Professional Prizes Honor Staff

New Harvard Features Service Goes Online

EXHIBIT C-6

Seven Students Win Paine Fellowships

Dental Center's Faculty Practice What They Teach

New Arteries Grown In Diseased Hearts

By William J. Cromie

Gazette Staff

Almost anything Hugh Curtis did gave him a pain in the heart. Even when lying in bed, he felt the stabbing chest pains of angina, a hurtful signal that his heart was not getting enough oxygen.

Curtis underwent a quadruple bypass in 1986, then a single bypass late last year. Surgeons removed veins from his legs and grafted them onto his heart to bypass his blocked coronary arteries. But that didn't solve his problem.

He also received a series of angioplasties, wherein tiny balloons were threaded into his heart's arteries, then inflated. This process pushed the blockages aside, opening his arteries. Five pieces of metal mesh were installed to keep them open, but his coronary arteries closed in other places.

"I couldn't walk very far, couldn't even make my bed," says the 55-year-old resident of Danvers, Mass. "Climbing stairs was out, so was any thought of going on vacation."

Late last year, he was asked by researchers at Beth Israel Deaconess Medical Center in Boston if he wanted to volunteer for an experimental procedure at the Harvard-affiliated hospital. The procedure involved doctors injecting proteins called growth factors into his heart to stimulate growth of new blood around those clogged with plaque.

"I didn't hesitate to give them the go-ahead," Curtis recalls.

The cardiologists threaded a thin hollow tube from his groin into his heart. Through the tube they injected what is called basic fibroblast growth factor, or bFGF.

Four months after the treatment, Curtis is back working full time at a desk job in a printing company. "I no longer take 3-to-6 nitroglycerin tablets a day, and I'm painting the hallway in my house," he says cheerily. "I may never go back to playing racquetball, but I'm leading a normal life, and that's all I'm looking for."

"All his symptoms are gone," says Michael Simons, associate professor of medicine at Harvard Medical School. "He is one of 18 patients who participated in a trial of bFGF. All are now largely without

symptoms such as chest pain, shortness of breath, and fatigue."

Bypassing Bypass Surgery

Eighteen other patients who received heart-artery bypasses got bFGF at the same time. Frank Sellke, an associate professor of surgery at Harvard Medical School, implanted capsules that slowly release the drug at sites where blocked vessels were too small or too diffusely diseased to bypass.

"These patients have undergone treadmill stress tests," Simons comments. "They also have been examined with a new type of magnetic resonance imaging (MRI) that measures blood flow and detects new vessel development. It is too early to scream and shout with success, but we are pleased with the results obtained so far."

"I had an MRI a couple of weeks ago, and it showed new arteries growing and bypassing some blockage," says Curtis. "I'm getting 70 percent blood flow to an area of the heart that was down to 30 percent flow. And there's reason to think things will improve more with time."

John Modugno, 48, received bFGF in February, and his MRI tests also show evidence of new arterial growth. "I feel much better," he says, "although I'm still on drugs and get a little angina at the end of the day."

Tests of bFGF and other growth factors now under way at various research centers raise hopes that newly grown blood vessels will replace arteries choked off by atherosclerosis, thus heading off thousands, maybe millions, of heart failures and heart attacks.

If these tests continue to be successful in humans, they could lead to heart drugs that will be cheaper, safer, and a lot easier on patients than bypass surgery and angioplasty. About a million people undergo such procedures in the United States each year, but they don't always work. As in Hugh Curtis's case, some vessels are too small or located where they can't be bypassed with sections of vein. After arteries have been opened by an inflated balloon or other types of angioplasty, about one-third of them close again, some in a matter of months.

"We once thought people in which neither procedure worked accounted for only a small subgroup of patients," Simons says. "But now we're getting phone calls almost every day, so we must conclude that there are more people with this problem than we imagined."

The revolutionary potential of growth factors, of course, goes far beyond such people. Simons sees it as "having the potential to replace or reduce the use of bypass surgery." The American Heart Association estimates that 500,000 bypasses are performed each year at an average cost of $45,000 per treatment.

Severely blocked coronary arteries cause more than 3 million heart failures a year, and 7 million more people suffer the chest pains of angina. "Growth-factor treatments might be expanded to many, if not all, of these patients," Simons declares.

The Side-Effects Question

Researchers at Beth Israel Deaconess Medical Center initiated such treatments in 1996. Today, seven

teams worldwide work on growing new blood vessels with bFGF and another protein known as vascular endothelial growth factor, or VEGF (see April 23 *Gazette*, page 1).

In a trial conducted at several medical centers, VEGF was given to 17 people whose blocked coronary arteries lay out of reach of angioplasty. Fifteen of the 17 patients showed various levels of improvement.

Jeffrey Isner, a cardiologist at St. Elizabeth's Medical Center in Boston, has used VEGF to grow new vessels around blockages in the leg veins of diabetics. He has treated 30 diabetic patients, as well as five other patients with heart disease.

"Preliminary results look good in both types of disease," Isner says. "This is a very encouraging and exciting area of treatment."

The great promise of bypassing blood-vessel blockages won't become a reality, however, if the growth factors cause severe side effects.

Both bFGF and VEGF lower blood pressure. "That fact limits the amount you can give a person," Simons notes. "But that's something we can work around."

More serious is the possibility of damage to sight caused by overgrowth of blood vessels in the eye. "We have been looking carefully for this, but have not seen any as yet with bFGF," Simons comments. Also, no new blood vessels were seen growing in the eyes of patients treated with VEGF, another encouraging sign.

The most worrisome possibility concerns growth of blood vessels that might nourish small, hidden cancer tumors. Judah Folkman, another Harvard researcher, found that such tumors remain benign unless new blood vessels carry nutrients to them. Once connected to a steady blood supply, tumors grow and spread.

Folkman and Michael O'Reilly developed two exciting new cancer drugs, endostatin and angiostatin, which block rather than promote development of blood vessels.

"We hope that tumor growth can be avoided because we give the growth factor for a very short time and in small amounts," Simons notes. "It's not like we're adding a foreign substance to the body; everyone has such small amounts of bFGF circulating naturally in their bloodstream."

The side-effects issue will be addressed in tests involving larger numbers of patients. Plans call for testing both growth factors on 400 to 500 people at a combination of medical centers throughout the country. Simons expects to start expanded trials of bFGF this summer in a collaboration with Emory University in Atlanta.

A question still to be answered is exactly how new blood vessels form. The bare-bones explanation has bFGF binding to the surface of, then stimulating growth of endothelial cells, those that line the inside of capillaries, the smallest vessels. These cells leave the vessels, migrate to the tip of the capillaries, and form a tube that extends their reach.

Simons's team took startling photos of new vessels growing around blocked arteries in animals. They show small extensions sprouting like twigs on a tree limb, moving around the barricade and reconnecting on the other side.

"It's amazing to see," Simons says. "If we can continue to do the same thing in humans, without deleterious side effects, we have a chance to benefit millions of people."

END

College Admission Yield Is Nearly 80%

Highest in 25 years

Nearly 80 percent of students admitted to the Class of 2002 have chosen to enroll, the highest yield since the early 1970s, according to the Undergraduate Admissions Office. This yield is the best in more than 25 years.

Yield, the percentage of admitted candidates who decide to accept an offer of admission, is considered a measure of a school's competitiveness. Harvard's yield is again, by a wide margin, the highest of the nation's selective colleges. When the final figures are available, the yield could go even higher -- it is already well above last year's yield of 76.3 percent.

The 2,073 students admitted to the Class of 2002 were selected from a pool of 16,819 applicants. For the seventh time in eight years, applications for admission to Harvard and Radcliffe have risen. Last year, 16,597 students applied for the 1,650 places in the entering class.

The substantial rise in the yield means that the Class of 2002 is now full, and it will probably be impossible to admit anyone from the waiting list. In more typical years, the College has been able to admit between 50 and 100 from the waiting list.

"We are extremely pleased that the College has again attracted so many extraordinarily talented students this year," said William R. Fitzsimmons '67, Dean of Admissions and Financial Aid. "With many leading American and international universities recently announcing changes in their financial aid programs designed to compete more aggressively for top students, the leadership of Dean of the Faculty of Arts and Sciences Jeremy Knowles and President Neil Rudenstine allowed Harvard to extend its best welcome to prospective members of the Class of 2002."

The Dean and President reemphasized their unwavering commitment to a strong need-based financial aid program and to the policy of admitting the best students without regard to their financial circumstances. With nearly 70 percent of all undergraduates on financial aid, and with scholarship grants of $45 million, Harvard has always been a leader in financial aid.

Dean Knowles stated in February, "We shall set no limit on the financial resources necessary to make Harvard College fully accessible to all students of promise. . . Students who are admitted to next fall's entering class will receive competitively supportive offers, and financial aid will be tailored flexibly and individually."

James S. Miller, director of financial aid, and his staff were available weekdays from 8 a.m. to 8 p.m. and several Saturdays for the month of April, and talked with an unprecedented number of students and parents about their financial aid awards. "Jim and his staff worked extremely hard to make it possible for

EXHIBIT C-7

Early reports

Clinical evidence of angiogenesis after arterial gene transfer of phVEGF₁₆₅ in patient with ischaemic limb

Jeffrey M Isner, Ann Pieczek, Robert Schainfeld, Richard Blair, Laura Haley, Takayuki Asahara, Kenneth Rosenfield, Syed Razvi, Kenneth Walsh, James F Symes

Summary

Background Preclinical findings suggest that intra-arterial gene transfer of a plasmid which encodes for vascular endothelial growth factor (VEGF) can improve blood supply to the ischaemic limb. We have used the method in a patient.

Methods Our patient was the eighth in a dose-ranging series. She was aged 71 with an ischaemic right leg. We administered 2000 µg human plasmid phVEGF₁₆₅ that was applied to the hydrogel polymer coating of an angioplasty balloon. By inflating the balloon, plasmid DNA was transferred to the distal popliteal artery.

Findings Digital subtraction angiography 4 weeks after gene therapy showed an increase in collateral vessels at the knee, mid-tibial, and ankle levels, which persisted at a 12-week view. Intra-arterial doppler-flow studies showed increased resting and maximum flows (by 82% and 72%, respectively). Three spider angiomas developed on the right foot/ankle about a week after gene transfer; one lesion was excised and revealed proliferative endothelium, the other two regressed. The patient developed oedema in her right leg, which was treated successfully.

Interpretation Administration of endothelial cell mitogens promotes angiogenesis in patients with limb ischaemia.

Lancet 1996; 348: 370–74

Introduction

Among the growth factors that promote angiogenesis, vascular endothelial growth factor (VEGF),[1] also known as vascular permeability factor,[2] and vasculotropin,[3] is specifically mitogenic for endothelial cells. The first exon of the VEGF gene includes a secretory signal sequence that permits the protein to be secreted naturally from intact cells.[3] We have shown[4,5] that arterial gene transfer of naked DNA encoding for secreted protein yielded physiological levels of protein despite low transfection efficiency. Site-specific gene transfer of plasmid DNA encoding the 165-aminoacid isoform of human VEGF (phVEGF₁₆₅) applied to the hydrogel polymer coating of an angioplasty balloon,[6] and delivered percutaneously to the iliac artery of rabbits in which the femoral artery had been excised to cause unilateral hindlimb ischaemia led to

Departments of Medicine, Biomedical Research, Radiology, and Surgery, St Elizabeth's Medical Center, Tufts University School of Medicine, Boston, Massachusetts, USA (Jeffrey M Isner MD, Ann Pieczek RN, Robert Schainfeld DO, Richard Blair MD, Laura Haley RN, Takayuki Asahara MD, Kenneth Rosenfield MD, Syed Razvi MD, Kenneth Walsh PhD, James F Symes MD)

Correspondence to: Dr Jeffrey M Isner, St Elizabeth's Medical Center, Boston, MA 02135, USA

development of collateral vessels and increased capillary density, improved calf blood-pressure ratio (ischaemic/normal limb) and increased resting and maximum vasodilator-induced blood flow.[5,6] We now use this strategy in the ischaemic limb of a patient.

Patient and methods

Patient

A 70-year-old non-diabetic woman was referred for gangrene of the right great toe. About a year earlier, the patient had cramping right-foot pain; several corns were removed, she was given intramuscular cortisone, prescribed ibuprofen, and fitted with shoe inserts. Symptoms worsened and the patient received oxycodone, hydrocodone, and a fentanyl patch. The great toe lesion progressed to gangrene, and the second and third toes became compromised. She had no palpable pedal pulses of the right limb. Ankle-brachial index of the ischaemic limb was 0·26. Arteriography revealed a 40% stenosis of the proximal popliteal artery, and occlusion of the peroneal, anterior tibial, and posterior tibial arteries midway to the foot. Surgical exploration of the distal right limb failed to identify a suitable site for a bypass.

The patient was suitable for arterial gene therapy according to a protocol[14] approved by the Human Institutional Review Board and Institutional Biosafety Committee of our centre, as well as the Recombinant DNA Advisory Committee of the National Institutes of Health and the US Food and Drug Administration.

Plasmid DNA

phVEGF₁₆₅ consists of a eucaryotic PUC 118 expression vector into which cDNA encoding the 165-aminoacid isoform of VEGF has been inserted.[15] A 763 basepair cytomegalovirus promoter/enhancer is used to drive VEGF expression. The PUC 118 vector includes an SV40 polyadenylation sequence, an *Escherichia coli* origin of replication, and the β-lactamase gene for ampicillin resistance. The plasmid was prepared in the Human Gene Therapy Laboratory at our centre from cultures of phVEGF₁₆₅-transformed *E coli*, purified with a Qiagen-tip 2500 column, precipitated with isopropanol, washed with 70% ethanol, and dried on a Speed Vac. The purified plasmid was reconstituted in sterile saline, stored in vials, and pooled for quality control analyses (absorbance at wavelengths of 260 and 280 nm to document ratio between 1·75 and 1·85; limulus amoebocyte lysate gel-clot assay [BioWhittaker] to establish bacterial endotoxin levels below 5 endotoxin units per kg bodyweight; microbial cultures; southern blot for level of contaminating genomic *E coli* DNA; and ethidium bromide staining after agarose-gel electrophoresis to confirm that over 90% of the nucleic acid was in the closed, circular supercoiled form). To confirm the identity of the prepared plasmid, the VEGF-coding region from each pooled batch was resequenced (Applied Biosystem 373A).

Percutaneous arterial gene transfer

Arterial gene transfer was done with a hydrogel-coated balloon-angioplasty-catheter (Boston Scientific).[6] A sterile pipette was used to apply 2000 µg plasmid DNA at 10·3 µg/µL in 194·2 µL

EXHIBIT D

DISCLOSURES

EVIDENCE APPENDIX

ITEM NO. 3

Declaration of Dr. Andrew E. Lorincz cited by Appellant as an
Exhibit in the Amendment filed February 15, 2001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: James P. Elia)
)
SERIAL NO.: 09/064,000) EXAMINER: Nicholas D. Lucchesi
)
FILED: April 21, 1998)
) GROUP ART UNIT: 3732
FOR: METHOD AND APPARATUS)
FOR INSTALLATION OF)
DENTAL IMPLANT)

DECLARATION OF ANDREW E. LORINCZ, M.D.

I Andrew E. Lorincz declare as follows:

1. I reside at 3628 Belle Meade Way, Mountain Brook, Alabama 35223.

2. My Curriculum Vitae is attached hereto as Exhibit A.

3. I have read and understood the disclosures at column 14, lines 4-61 and column 21, lines 1-26 of United States Patent Number 5,397,235 (hereinafter " '235 patent") entitled "Method for Installation of Dental Implant," and granted to James P. Elia on March 14, 1995. A copy of such disclosures is attached hereto as Exhibit D. I understand that the same disclosures are contained in above patent application Serial No. 09/064,000.

4. I note that the disclosures mentioned in above Paragraph 3 relate to a method for forming a bud and then for forming soft tissue. Such methods involve placing a growth factor at a desired site of a body with use of techniques including resorbable and non-resorbable carriers, gels, time-

1

release capsules, and granules. In addition, the growth factor may be placed in the body orally, systemically, by injection, through the respiratory tract, by making an incision in the body and then inserting the growth factor. I note further that the growth factor and/or carrier may be activated by tissue pH, enzymes, ultrasound, electricity, heat, or in vivo chemicals.

5. It is well known and established in the medical arts that buds are a primordium or, in other words, a rudiment or commencement of an organ. The process of organ formation includes the differential development of cells to form an organ primordium with the resulting formation of soft tissue. Such process of development is called organogenesis. It is also well known and established in the medical arts that the term "soft tissue" includes blood vessels.

    In making the above statement in this Paragraph, I am aware of the definitions attached hereto as Exhibit B. Terms included in the above-mentioned definitions are: bud, primordium; organogenesis, and organ. I am also aware of and have considered the definition of "growth factor" as contained in Column 14 of the aforesaid '235 patent.

6. The materials included in attached Exhibit C evidence that the placement of growth factors in the body of a human results in the formation of a bud with subsequent growth into soft tissue. These materials report work performed by reputable, skilled scientists and reputable organizations in the medical arts. Consequently, I believe that these reports would be recognized as clearly valid by one of ordinary skill in the medical arts because they report the results of scientific tests conducted by competent, disinterested third parties with use of proper scientific controls.

2

7.   Based upon the materials included in above Paragraphs 4, 5, and 6, it is my opinion that the process of placing a growth factor at a desired site of a human body will produce a bud that will predictably subsequently grow into soft tissue, as described in the '235 patent, using the techniques identified in above Paragraph 4. My further opinion is that when the techniques and angiogenic growth factors described and disclosed in the Elia patent application are used to place such growth factors in a human host, such placement would result in the formation of soft tissue, e.g., blood vessels. My opinion is in accord with the results obtained by the Isner patent (Exhibit C-6) which employed the same angiogenic growth factors and carrier/technique described and disclosed in the Elia patent application.

8.   Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: ___2-12-01___

_____
Andrew E. Lorincz

EXHIBIT A

CURRICULUM VITAE

NAME: Andrew E. Lorincz, M.D.

TITLE: Professor of Pediatrics
                         University of Alabama at Birmingham

SOCIAL SECURITY: 361-16-4853

ADDRESSES: University of Alabama at Birmingham
                         Mortimer Jordan Hall B-70
                         1825 University Boulevard
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                         Mountain Brook, Alabama 35223
                         Telephone: (205) 967-4678

BIRTH: 5/17/26 Chicago, Illinois

MARITAL STATUS: Married, 12/14/65 - Diane DeNyse Lorincz

EDUCATION:

1948-1952   University of Chicago, School of Medicine, M.D. Degree
1948-1950   University of Chicago, B.S. Degree (Anatomy & Biochemistry)
1946-1948   University of Chicago, Ph.B. Degree

POSTDOCTORAL EDUCATION:

Jan-Mar 1980   Lysosomal Storage Disease Laboratory, Eunice Kennedy Shriver Center,
                 Waltham, MA (Harvard), Visiting Scientist
1955-1956   LaRabida Jackson Park Sanitarium, University of Chicago,
                     Junior Staff Physician Department of Pediatrics, University of Chicago
                 Clinics Bob Roberts Memorial Hospital
1955-1958   Arthritis and Rheumatism Foundation Fellow
1954-1955   Benjamin J Rosenthal Clinical and Research Fellow
1953-1954   Junior Assistant Resident
1952-1953   Intern

ACADEMIC APPOINTMENTS:

1996-present	Professor Emeritus, Department of Pediatrics
1984-1996	School of Public Health, University of Alabama at Birmingham, Professor
1971-1996	University of Alabama at Birmingham, Member of Graduate Faculty
1968-1996	University of Alabama at Birmingham, Professor of Pediatrics
1971-1984	Division of Engineering Biophysics, University of Alabama at Birmingham, Associate Professor
1968-1982	University of Alabama at Birmingham, Associate Professor of Biochemistry
1976-1980	School of Optometry, University of Alabama at Birmingham, Professor Optometry
1971-1980	School of Nursing, University of Alabama at Birmingham, Clinical Associate Professor
1970-1980	Center for Developmental and Learning Disorders, University of Alabama at Birmingham, (A University Affiliated Facility for Developmental Disability), Director
1970-1976	School of Optometry, University of Alabama at Birmingham, Associate Professor of Pediatric Optometry
1966-1968	Medical Teaching and Research, Unit of the University of Florida at the Sunland Training Center, Gainesville, Florida, Director
1963-1968	Department of Surgery (Orthopaedics), University of Florida College of Medicine, Gainesville, Florida, Research Associate Professor
1962-1968	Department of Pediatrics, University of Florida College of Medicine, Gainesville, Florida, Associate Professor
1959-1962	Department of Pediatrics, University of Florida College of Medicine, Gainesville, Florida, Assistant Professor
1956-1959	Department of Pediatrics, University of Chicago School of Medicine, Chicago, Illinois, Instructor

PROFESSIONAL LICENSES - PHYSICIAN AND SURGEON:

5/26/69	State of Alabama
8/10/59	State of Florida (inactive)
9/22/54	State of Illinois (inactive)

SPECIALTY CERTIFICATION:

May 1958   American Board of Pediatrics, Diplomate

BOARDS, COMMITTEES AND CONSULTANTSHIPS:

1994-present	Board member of The Mental Retardation and Developmental Disabilities, Health Care Authority of Jefferson County, Inc.
1991-present	Editorial Board for the Annals of Clinical and Laboratory Science, Member
1988-present	Medical Advisory Board of the National MPS Society, Member
1980-1986	Mental Retardation, Consulting Editor
1979-present	National Tay-Sachs and Allied Diseases Association, Scientific Advisory Committee, Member
1978-present	Mayor's Council of Disability Issues
1979-1984	Osteogenesis Imperfecta Foundation, Inc., Board Member Alabama O.I.Chapter
1974-1981	Child Mental Health Services, Inc., Birmingham, Alabama, Board Member
1977-1978	Elizabethtown Committee on Planning and Evaluation, Legislative Committee, State of Pennsylvania
1973-1975	Human Rights Committee for the Partlow State School and Hospital, Tuscaloosa, Alabama, Member - Federal Court Appointed
1971-1974	American Academy of Pediatrics, Committee on Children With Handicaps
1971-1973	American Journal of Mental Deficiency, Consulting Editor
1965-1973	Head Start, Medical Consultant
1967-1972	Journal of Investigative Dermatology, Editorial Consultant
1961-1968	Sunland Hospital, Orlando, Florida, Medical and Research Consultant
1965-1966	State of Florida Interagency Committee on Mental Retardation Planning, Co-Chairman, Mental Retardation Research Committee Alabama Developmental Disabilites Planning Council
1982-1984	Maternal and Child Health, Member of Advisory Committee
1979-1984	Member (Secretary, 1980; Vice Chairman, 1984)
1973-1979	Consultant

American Association of University Affiliated Facilities

1975-1978	American Association of University Affiliated Programs for the Developmentally Disabled, Board Member

American Association on Mental Retardation

1980,84,85	Prevention Committee, Chairman
1980-1982	Member of Council
1978-1980	Medicine Division and Member Executive Committee, Vice President

BOARDS, COMMITTEES AND CONSULTANTSHIPS: (CONT'D)

Association of Retarded Citizens of Jefferson County

1990-present	Board Member
1975-1985	Board Member
1977-1978	Second Vice President
1980	Recipient of Distinguished Service Award

PROFESSIONAL SOCIETIES:

American Academy for Cerebral Palsy and Developmental Medicine, (Fellow)
American Academy on Mental Retardation (President Elect, 1975-76; President, 1976-77)
    Emeritus Member
American Academy of Pediatrics (Fellow)
American Association for the Advancement of Science
American Association for Clinical Chemistry, Inc.
American Association on Mental Retardation (Fellow)-Life Member
American Chemical Society
American Federation for Clinical Research
American Medical Association
American Society for Human Genetics
American Society for Investigative Pathology
Association of Clinical Scientists
International Society for Mycoplasmology
Jefferson County Pediatric Society
Society for Complex Carbohydrates
Society for Investigative Dermatology
Society for Pediatric Research
Society for Sigma Xi
Southern Society for Pediatric Research (President, 1964)

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2. Dorfman, A., Gross, J.I.; Lorincz, A.E.: The Treatment of Acute Rheumatic Fever. Pediatrics, 27:692-705, May, 1961.

3. Lorincz, A.E.: Heritable Disorders of Acid Mucopolysaccharide Metabolism in Humans and in Snorter Dwarf Cattle. Annals of the New York Academy of Science, 91:644-58, 1961.

4. Shepard, T.H., Lorincz, A.E., Gartler, S.M.: Desulfuration of Thiourea by Saliva. Proceedings of the Society of Experimental Biology and Medicine, 112:38-42, July, 1963.

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6. Gennaro, J.F., Jr., Callahan, W.P., III, Lorincz, A.E.: The Anatomy and Biochemistry of a Mucus-secreting Cell Type Present in the Poison Apparatus of the Pit Viper, Ancistrodon Piscivorus Piscivorus. Annals of the New York Academy of Sciences, 106:463-71, March, 1963.

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8. Wainer, A., Lorincz, A.E.: Studies of Mercapturic Acid Synthesis by Humans. Life Sciences, No.7:504-08, 1963.

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12. Lorincz, A.E.: Hurler's Syndrome. Medical Aspects of Mental Retardation, C.H. Carter (Editor), Chapter 19, pp. 628-650, 1965.

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4. Lorincz, A.E.: "Snorter" Dwarf Cattle: A Naturally Occurring Heretable Disorder of Acid Mucopolysaccharide Metabolism Which Resembles Hurler Syndrome. American Journal of Diseases of Children, 100:488, 1960.

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7. Wolfson, S.L., Kahana, L., Harris, J.S., Lorincz, A.E.: Biochemical and Roetgenographic Response to Long-term Corticosteroid Therapy on the Hurler Syndrome. American Journal of Diseases of Children, 102:638, 1961.

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10. Gessner, L.H., Lorincz, A.E.: Acid Mucopolysaccharide Content of the Three-day Old Chick Embryo Heart. Federation Proceedings, 22:2, December, 1963.

11. Krovetz, L.J., Lorincz, A.E., Schiebler, G.L.: Hemodynamic Studies in the Hunter-Hurler Syndrome (Gargoylism). Circulation, 28:753., 1963.

12. Batey, R.L., Lorincz, A.E.: A New Technique for the Identification of Acid Mucopolysaccharide Hydrolysates. Southern Medical Journal, 56:1437, 1963.

13. Callahan, W.P., Lorincz, A.E.: Changes in Liver Ultrastructure Associated With Hurler Syndrome. Anatomical Record, 148:267, 1964.

14. Renn, W.H., Lorincz, A.E.: A New Urinary Mucopolysaccharide Associated With the Nail-Patella Syndrome (Hereditary arthro-osteo-onychodydysplasia). Southern Medical Journal, 58:1584, December, 1965.

15. Lorincz, A.E., Callahan, W.P.: Electron Microscopic Observations of Liver in the Hurler Syndrome. Southern Medical Journal, 58:1584, December, 1965.

16. Renn, W.H., Enneking, W.F., Hase, M.F., Lorincz, A.E.: Acid Mucopolysaccharide Content of Human Chondrosarcomatous Tumors. Journal of Bone and Joint Surgery, 50-A:1072, 1968.

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18. Lorincz, A.E., Finley, S.C., Finley, W.H., West, S.S.: Biophysical Cytochemical Study of Glycosaminoglycans in Fibroblasts and Other Cells Derived From Marteaux-Lamy Mucopolysaccharidosis. Federation Proceedings, Vol. 11, No. 2, March-April, 1973.

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20. Lorincz, A.E., West, S.S.: The Biophysical Cytochemistry of Living Cells Derived From Patients With Mucopolysaccharidoses. Journal of Bone and Joint Surgery. Proceedings of the 19th Annual Meeting of the Orthopedic Research Society, Las Vegas, Nevada, 55-A:655, January 30, 31 & February 1, 1973.

21. Hurst, R.E., Cezayirli, R.C., Lorincz, A.E.: The Nature of the Glycosaminoglycans (Mucoploysacchariduria) in Brachycephalic "Snorter" Dwarf Cattle. Federation Proceedings, 34:861, March, 1975.

22. Lorincz, A.E., Hurst, R.E., Cezayirli, R.C.: Definitive Characterization of the Glycosaminoglycanuria in "Snorter" Dwarf Cattle. Pediatric Research, 9:314, 1975.

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25. Lorincz, A.E.: Direct Visualization of Mycoplasma via Supravital Staining and Fluorescence Microscopy. Abstracts of the 6th International Congress of the International Organization for Mycoplasma, p. 272, 1986.

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EDITORIALS AND COMMENTARIES

1. Lorincz, A.E.: Biochemical Genetic Defects. Journal of the Florida Medical Association, 66:852, 1960.

2. Lorincz, A.E., Smith, R.T.: Research at the University of Florida College of Medicine, Department of Pediatrics. Journal of the Florida Medical Association, 51:808-11, 1965.

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BOOK REVIEWS

1. "Modern Topics in Paediatric Dermatology", Verbov, J. (Editor), J.B. Lippincott, Philadelphia (Publisher), JAMA, 244:1269, 1980.

2. "Dermatologic Disorders in Black Children and Adolescents", Laude, T.A. & Russo, R.U. (Editors), Medical Examination Publishing Company, New Hyde Park, New York (Publisher), JAMA, 252:1769-70, 1984.

3. "Practical Pediatric Dermatology", Weston, W.L. (Editor), Little Brown and Company, Boston (Publisher), JAMA, 277:549, 1987.

EXHIBIT
B

DEFINITIONS

STEDMAN'S
MEDICAL
DICTIONARY

ILLUSTRATED

*A vocabulary of medicine and
its allied sciences, with pronunciations
and derivations*

TWENTY-SECOND EDITION

*Completely revised by a staff of 33 editors, covering
44 specialties and subspecialties*

anxiety accompanying psychosomatic disorders; should not be used for nausea of pregnancy.

bud. A structure that resembles the b. of a plant.

bronchial b., one of the outgrowths from the primordial bronchus responsible for the continued ramification of the embryonic bronchial tree.

end b., tail b.

farcy b., one of a number of nodules formed along the course of the subcutaneous lymphatics in cases of glanders.

gustatory b., *caliculus* gustatorius.

liver b., the primordial cellular outgrowth from foregut entoderm of the embryo that gives rise to the parenchyma of the liver.

lung b., in the embryo, one of the two lateral outgrowths from the respiratory primordium that ultimately forms the epithelial portions of the lung.

metanephric b., ureteric b.; the primordial cellular outgrowth from the mesonephric duct that gives rise to the epithelial lining of the ureter, pelvis and calyces of the kidney, and the straight collecting tubules.

syncytial b., syncytial *knot*.

tail b., end b.; the rapidly proliferating mass of cells at the caudal extremity of the embryo.

taste b., *caliculus* gustatorius.

tooth b., the primordial structures from which a tooth is formed; the enamel organ, dental papilla, and the dental sac enclosing them.

ureteric b., metanephric b.

vascular b., an endothelial sprout arising from a blood vessel.

Budd, George, London physician, 1808–1882. See B.'s *cirrhosis, jaundice, syndrome.*

Budde (bood'deh), E., Danish sanitary engineer, *1871. See B. *process.*

buddeize (bood'de-ize). To treat by the Budde process.

budding. Gemmation.

Budge (bood'ga), Julius L., German physiologist, 1811–1888. See B.'s *center.*

Budin (bü-dän'), Pierre C., French gynecologist, 1846–1907. See B.'s obstetrical *joint,* B.'s *pelvimeter.*

Buerger, Leo, New York physician, 1879–1943. See B.'s *disease,* Winiwarter-B. *disease,* B.'s *stain.*

bufa-, bufo-. Combining forms that denote origin from toads. They are used in the systematic and trivial names of a great number of toxic substances (genins) isolated from plants and animals containing the bufanolide structure (see bufanolide). Prefixes denoting species origin are often attached, *e.g.,* marinobufagin, marinobufotoxin.

bufagenins. Bufagins.

bufagins. Bufagenins: a group of steroids (bufanolides) in the venom of a family of toads, the Bufonidae, having a digitalis-like action upon the heart (*e.g.,* bufotalin); *cf.* bufotoxins. For structure of bufanolides. see steroids.

bufalin. A specific type of bufanolide, containing 3β,14-dihydroxy-5β,14β-bufa-20,22-dienolide. For structure of bufanolide, see steroids.

bufanolide. Fundamental steroid lactone of several squill-toad (Bufonidae) venoms or toxins; also found in the form of glycosides in plants (*cf.* digitalis). The steroid is essentially that of 5β-androstane, with a 14β-H. The lactone at C-17 is structurally related to —CH(CH₃)CH₂CH₂CH₃ radical attached to C-17 in the cholanes, and is in the same configuration as that of cholesterol (*i.e.,* 20 R); in some species, b. is formed from cholesterol. Various b. derivatives having unsaturation in the lactone ring (20,22) are known as bufenolides (one double bond), bufadienolides (*e.g.,* bufalin, telecinobufagin, marinobufagin, bufogenin B, bufotalin, bufotoxin), bufatrienolides (*e.g.,* scillarenin), etc. They have varying numbers of hydroxyl groups at positions 3, 5, 14, and 16, and these may be further substituted (*e.g.,* bufatalin, bufotoxin, gitoxigenin). For structure, see steroids.

buffer. 1. A mixture of an acid and its conjugate base (salt), such as H₂CO₃/HCO₃⁻; H₂PO₄⁻/H₂PO₄⁻, which when present in a solution reduces any changes in pH that would otherwise occur in the solution when acid or alkali is added to it. Thus the pH of the blood and body fluids is maintained virtually constant (pH 7.45)

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although acid metabolites are continually being formed in the tissues and CO₂(H₂CO₃) is lost in the lungs. See also conjugate acid-base pair, under conjugate. 2. To add a b. to a solution and thus give it the property of resisting a change in pH when it receives a limited amount of acid or alkali.

b. capacity, the amount of hydrogen ion (or hydroxyl ion) required to bring about a specific pH change in a specified volume of a b. (see b. value).

b. pair, an acid and its conjugate base (anion).

secondary b., see Hamburger's *law.*

b. value, the power of a substance in solution to absorb acid or alkali without change in pH; this is highest at a pH equal to the pK of the acid of the b. pair (see b. capacity).

b. value of the blood, the ability of the blood to compensate for acid-alkali fluctuations without disturbance of the pH.

Buffon (boo'-fon), Compt de (Georges Louis Leclerc), French naturalist, 1707–1788. Published *Histoire Naturelle.* Some of his views on evolution and the origin of species anticipated Darwin by more than a hundred years.

buffy coat. Crusta inflammatoria; crusta phlogistica; the upper, lighter portion of the blood clot (coagulated plasma and white blood cells), occurring when coagulation is delayed so that the red blood cells have had time to settle a little; the portion of centrifuged, anticoagulated blood which contains leukocytes and platelets.

bufo-. See bufa-.

bufogenin B. A steroid toxin from Chinese toads; a 3β,14,16-trihydroxy-bufa-20,22-dienolide; *cf.* bufalin.

Bufonidae [L. *bufo,* toad]. A family of toads whose dermal glands secrete several kinds of pharmacologically active substances having a cardiac action similar to that of digitalis.

bufotalin. The steroid of a bufotoxin (bufogenin). It is bufogenin B acetylated at the C-16 OH.

bufoten'ine. Mappine; 3-(2-dimethylaminoethyl)indol-5-ol; N,N-dimethylserotin; a psychotomimetic agent isolated from the venom of certain toads. It raises the blood pressure by a vasoconstrictor action and produces psychic effects including hallucinations. It is also present in several plants and is one of the active principles of cohoba.

bufotox'in. Vulgarobufotoxin; a toxic substance in venom of *Bufa vulgaris,* the common European toad; bufotalin esterified with suberylarginine at C-14 OH group.

bufotox'ins. A group of steroid lactones (conjugates of bufogenins and suberylarginine at C-14) of digitalis present in the venoms of the Bufonidae. Their effects are similar to but weaker than those of the bufagins.

bug'gery [O.F. *bougre,* heretic]. Bestiality; sodomy.

Buhl (bool), Ludwig von, German pathologist, 1816–1880. See B.'s *disease.*

Buist, Robert C., Scottish obstetrician, 1860–1939. See B.'s *method.*

bulb [L. *bulbus,* a bulbous root]. 1. Any globular or fusiform structure. 2. *Medulla* oblongata. 3. A short, vertical underground stem of plants such as scilla and allium.

aortic b., *bulbus* aortae.

arterial b., *bulbus* aortae.

carotid b., *sinus* caroticus.

b. of corpus spongiosum, *bulbus* penis.

dental b., the papilla, derived from mesoderm, that forms the part of the primordium of a tooth which is situated within the cup-shaped enamel organ.

duode'nal b., duodenal *cap.*

end b., one of the oval or rounded bodies in which the sensory nerve fibers terminate in mucous membrane.

b. of eye, *bulbus* oculi.

hair b., *bulbus* pili.

ju'gular b., *bulbus* venae jugularis.

Krause's end b., *corpusculum* bulboideum.

b. of lateral ventricle, a rounded elevation in the dorsal part of the medial wall of the posterior horn of the lateral ventricle, produced by the forceps major.

olfac'tory b., *bulbus* olfactorius.

b. of penis, *bulbus* penis.

rachid'ian b., *medulla* oblongata

Rouget's b., a venous pl[...] taste b., *caliculus* gustato[...] b. of ure'thra, *bulbus* pe[...] b. of vestibule, *bulbus* v[...]

bulbar. 1. Relating to a bull[...] medulla oblongata.

bulbi'tis. Inflammation of [...]

bulbocap'nine [G. *bolbos,* [...] An alkaloid from *Corydalis* [...] *Fumariaceae).* Produces a s[...] recommended in the treatmen[...] disease, paralysis agitans, an[...]

bul'bocaverno'sus. See u[...]

bulboid [G. *bolboeidés,* fr[...] resemblance]. Bulb-shaped.

bulbonu'clear. Relating to [...]

bulbopon'tine. Denoting [...] pons and the region of the m[...]

bulbosac'ral. Relating to [...] segments of the spinal cord.

bulbospin'al. Relating to th[...] particularly to nerve fibers in [...]

bulbourethral (bul'bo-u-re'[...]

bulbus, gen. and pl. bull[...] b. aor'tae [NA], aortic bult[...] dilation where the truncus arte[...]

b. cordis, b. aortae.

b. cornus posterior'is [N,[...] lateral ventricle of the brain; [...] of the posterior horn produce[...] of the corpus callosum as the [...] lobe.

b. oc'uli [NA], bulb of the [...] proper without the appendage[...]

b. olfacto'rius [NA], olf[...] anterior extremity of the olfac[...] plate of the ethmoid and rece[...]

Olfactory bulb

Cribriform plate of ethmoid

Olfactory mucosa

Bulbus O[...]

Diagram of olfactory muc[...] y Cajal), showing neuronal rel[...] Copenhaver, W. F.: *Bailey's* [...] The Williams & Wilkins Co., [...]

b. penis [NA], bulb of corp[...] b. urethrae; the expanded poster[...] penis lying in the interval betw[...]

b. pili [NA], hair bulb; the lo[...] that fits like a cap over the pap[...] follicle.

b. ure'thrae, b. penis.

b. venae jugula'ris [NA], b[...] dilated parts of the internal jugu[...] at the beginning of the internal j[...]

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│  Thesaurus
│          bud[1,noun]  ▲
│          bud[2,verb]  ▢
│  Go To   bud scale    ▼
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Main Entry: [1]**bud**
Pronunciation: 'b&d
Function: *noun*
Etymology: Middle English *budde*
Date: 14th century
1 : a small lateral or terminal protuberance on the stem of a plant that may develop into a flower, leaf, or shoot
2 : something not yet mature or at full development: as **a** : an incompletely opened flower **b** : CHILD, YOUTH **c** : an outgrowth of an organism that differentiates into a new individual : GEMMA; *also* : PRIMORDIUM
- **in the bud** : in an early stage of development in the bud>

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Thesaurus Symbol Key

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|| usage restricted; consult a dictionary for more information

For further explanation of these symbols see the Thesaurus Symbol Guide.

Dictionary Pronunciation Key

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Thesaurus

Main Entry: pri·mor·di·um
Pronunciation: -dE-&m
Function: *noun*
Inflected Form(s): *plural* **pri·mor·dia** /-dE-&/
Etymology: New Latin, from Latin
Date: 1671
: the rudiment or commencement of a part or organ

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Dictionary Pronunciation Key

- \&\ as a and ụ in abut
- \&\ as e in kitten
- \&r\ as ur and er in further
- \a\ as a in ash
- \A\ as a in ace
- \ä\ as o in mop
- \au\ as ou in out
- \ch\ as ch in chin

- \e\ as e in bet
- \E\ as ea in easy
- \g\ as g in go
- \i\ as i in hit
- \I\ as i in ice
- \j\ as j in job
- \[ng]\ as ng in sing
- \O\ as o in go

- \o\ as aw in law
- \oi\ as oy in boy
- \th\ as th in thin
- \th\ as th in the
- \ü\ as oo in loot
- \u\ as oo in foot
- \y\ as y in yet
- \zh\ as si in vision

Encyclopædia Britannica

organogenesis

organogenesis,

in embryology, the series of organized integrated processes that transforms an amorphous mass of cells into a complete organ in the developing embryo. The cells of an organ-forming region undergo differential development and movement to form an organ primordium, or anlage. Organogenesis continues until the definitive characteristics of the organ are achieved. Concurrent with this process is histogenesis; the result of both processes is a structurally and functionally complete organ. The accomplishment of organogenesis ends the period during which the developing organism is called an embryo and begins the period in which the organism is called a fetus. See also histogenesis.

(Merriam-Webster)

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    barrel organ
Go To  electric organ
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Main Entry: **organ**
Pronunciation: 'or-g&n
Function: *noun*
Etymology: Middle English, partly from Old English *organa*, from Latin *organum*, from Greek *organon*, literally, tool, instrument; partly from Old French *organe*, from Latin *organum*; akin to Greek *ergon* work -- more at WORK
Date: before 12th century
1 a *archaic* : any of various musical instruments; *especially* : WIND INSTRUMENT **b** (1) : a wind instrument consisting of sets of pipes made to sound by compressed air and controlled by keyboards and producing a variety of musical effects -- called also *pipe organ* (2) : REED ORGAN (3) : an instrument in which the sound and resources of the pipe organ are approximated by means of electronic devices (4) : any of various similar cruder instruments
2 a : a differentiated structure (as a heart, kidney, leaf, or stem) consisting of cells and tissues and performing some specific function in an organism **b** : bodily parts performing a function or cooperating in an activity organs>
3 : a subordinate group or organization that performs specialized functions organs of government>
4 : PERIODICAL

Dictionary Look Up: [   ] [Search]

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Thesaurus Symbol Key

* generally or often considered vulgar
|| usage restricted; consult a dictionary for more information

EXHIBIT C

EXHIBIT C
SUMMARY OF MATERIALS

EXH. NO.	MATERIAL AND DATE	SOFT TISSUE	TECHNIQUE	GROWTH FACTOR
C-1	Science Daily (American Heart Association), 1998, "Study is first ever to document protein therapy induces creation of new blood vessels to the human heart" SYNOPSIS: For the first time ever, growth factor inserted into the body grows a new vascular system.	Blood vessels to heart	Injection	Human recombinant basic fiberblast growth factor (genetically manipulated and produced)
C-2	Circulation, 1998, "Induction of neoangiogenesis in ischaemic myocardium by human growth factors: first clinical results of a new treatment of coronary heart disease" SYNOPSIS: A new therapeutic concept and followup tests confirm a true de novo vascular system was formed . Vascular buds consisting of endothelial sprouts (capillaries) were created. The capillaries grew further and differentiated into two-layered metarterioles. The process of organogenesis continued with the metarterioles differentiating into three-layered arterioles (arteries).	Blood vessels to heart	Injection	Human recombinant basic fiberblast growth factor (genetically manipulated and produced)

EXH. NO.	MATERIAL AND DATE	SOFT TISSUE	TECHNIQUE	GROWTH FACTOR
C-3	Circulation, 1998, Editorial, "Angiogenic therapy of the human heart" SYNOPSIS: Basic research in a different field (cancer) purified angiogenic growth factors in the 1980's. A novel clinical application of these growth factors introduces a new modality-the regulation of blood vessel growth.	Editorial	Editorial	Editorial
C-4	NIH Press Release: 1999, "Growing New blood vessels with a timed-release capsule of growth factor is a promising treatment for heart bypass patients, finds NHLBI Study" SYNOPSIS: Researchers at Harvard Mecial School inserted timed-release capsules of basic fibroblast growth factor into [human] heart muscle to grow new blood vessels.	Blood vessels to heart	Insertion of timed-release capsule	Basic fibroblast growth factor
C-5	The Lancet, 1996, "Clinical Evidence of angiogenesis after arterial gene transfer of phVEGF in Patient with Ischaemic limb" SYNOPSIS: Growth factor plus living material (plasmid) inserted into the body with a gel carrier to grow new blood vessels in the leg of a patient.	Blood vessels to leg	Balloon Catheter/ hydrogel	Vascular endothelial growth factor plus living material (plasmid)

2

EXH. NO.	MATERIAL AND DATE	SOFT TISSUE	TECHNIQUE	GROWTH FACTOR
C-6	U.S. Patent No. 5,652,225 (1997) Parent application filed 10/04/94 SYNOPSIS: The formation of new blood vessels in a human host by inserting a growth factor with a carrier into the body.	Formation of new blood vessels	Balloon catheter/ hydrogel	Angiogenic growth factors
C-7	Harvard University Gazette, 1998, "New Arteries Grown in Diseased Hearts" SYNOPSIS: Harvard Medical School researchers inject basic fibroblast growth factor through a carrier (tube) to grow new arteries in a human heart.	Formation of new arteries in hearts	Injection via tube (catheter); and implanted timed-release capsules	Basic fibroblast growth factor

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Source: American Heart Association (http://www.americanheart.org/)

Date: Posted 3/2/1998

Study Is First Ever To Document Protein Therapy Induces Creation Of New Blood Vessels To The Human Heart

DALLAS, Feb. 24 -- For the first time, scientists have published research evidence that recombinant protein therapy can create new blood vessels to increase blood supply to the human heart. The report from German scientists appears in today's Circulation: Journal of the American Heart Association.

FGF-I, a human growth factor obtained through genetic engineering, was used in 20 patients with some form of ischemic or coronary heart disease, which results from blockages in the vessels leading to and from the heart. By injecting the growth factor near the blocked vessels, the scientists were able to induce neoangiogenesis -- the process by which the body can grow its own new capillary network to bypass occluded vessels.

"This capillary network is a true de novo vascular system," says Thomas-Joseph Stegmann, M.D., head of the department of thoracic and cardiovascular surgery at the Fulda Medical Center, Fulda, Germany. "We were able to use the recognized physiological effects of FGF-I to induce neoangiogenesis in the human ischemic heart."

As early as four days after application of FGF-I, the vascular structure around the diseased vessels was completely altered in all 20 of the patients. Like the spokes of a bicycle wheel, the new capillary vessels radiated outward from the point of injection, resulting in a twofold to threefold increase in blood flow to the heart, says the study's lead author.

Researchers found, on average, the ejection fraction of the 20 patients improved from 50.3 percent to 63.8 percent in the three years following the procedure. Ejection fraction measures how much blood leaves the

EXHIBIT C-1

heart with each beat and indicates how well the left ventricle -- the heart's main pumping chamber -- is functioning.

In follow-up angiographic imaging of the patients, it was clear that the growth factor injection had stimulated the creation of a new vascular system, says Stegmann. Three months after the procedure, he and his colleagues examined angiograms -- X-ray images of the heart -- of both the treated and control (untreated) patients and found that no blockages had formed in the new vessels.

All of the patients who received the FGF-I three years ago are still alive. The scientists report that no negative side effects have been seen in the patients who received the FGF-I.

Elizabeth Nabel, M.D., an American Heart Association board member, has done extensive research in gene and recombinant protein therapy over the past 12 years. She says this new research is encouraging for cardiovascular surgeons.

"It's a very important therapy for patients who have blocked arteries that are not amenable to bypass," says Nabel, professor of internal medicine and physiology and chief, division of cardiology at the University of Michigan. "This is not to say that bypass should be abandoned, but this research shows angiogenesis is a powerful therapy to be used with bypass surgery."

The procedure is still experimental, but scientists say the use of FGF-I may particularly benefit patients whose blocked vessels cannot be treated by cardiac bypass operations.

"At the moment, this procedure could not replace conventional bypass surgery," says Stegmann. "The question remains to be answered whether FGF-I or other growth factors are able to treat occlusions of greater coronary vessels, but currently, this is not possible."

Scientists have used gene therapy to grow vessels in other parts of the body -- such as in the legs in order to improve the health of patients who have blockages in lower leg blood vessels -- but this is the first published account of the use of recombinant protein therapy to induce angiogenesis in human hearts.

FGF-I was obtained from strains of Escherichia coli by genetic engineering, then isolated and highly purified the recombinant FGF-I protein. After several series of animal experiments demonstrated the potency of FGF-I, it was used in humans for the first time.

When scientists create recombinant protein, they take the DNA of a growth factor (in this case FGF-I) and manipulate it into RNA (ribonucleic acid) by growing it in bacteria cultures in the laboratory. RNA is then manufactured into protein, which is isolated and purified

before it is injected into the hearts of patients.

Twenty patients — 14 men and 6 women who were at least 50 years old — who had no prior history of heart attack or cardiac surgery had an operation to clear blockages in more than one vessel. All of them had stenosis — narrowed blood flow due to atherosclerosis — in their internal mammary artery/left anterior descending coronary artery. During the operative procedure, the growth factor protein — in a dosage of 0.01 milligrams per kilogram of body weight — was directly injected into the heart muscle near the blockage.

Prior to using the treatment in humans, the scientists performed several series of animal experiments, most specifically in ischemic rat hearts. Having found that the FGF-I injection worked in those animal models, the researchers theorized that it would also work in humans.

Study co-authors are P. Pecher, M.D.; B.U. von Specht, M.D. and B. Schumacher, M.D.

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Induction of Neoangiogenesis in Ischemic Myocardium by Human Growth Factors

First Clinical Results of a New Treatment of Coronary Heart Disease

B. Schumacher, MD; P. Pecher, MD; B.U. von Specht, MD; Th. Stegmann, MD

Background—The present article is a report of our animal experiments and also of the first clinical results of a new treatment for coronary heart disease using the human growth factor FGF-I (basic fibroblast growth factor) to induce neoangiogenesis in the ischemic myocardium.

Methods and Results—FGF-I was obtained from strains of *Escherichia coli* by genetic engineering, then isolated and highly purified. Several series of animal experiments demonstrated the apathogenic action and neoangiogenic potency of this factor. After successful conclusion of the animal experiments, it was used clinically for the first time. FGF-I (0.01 mg/kg body weight) was injected close to the vessels after the completion of internal mammary artery (IMA)/left anterior descending coronary artery (LAD) anastomosis in 20 patients with three-vessel coronary disease. All the patients had additional peripheral stenoses of the LAD or one of its diagonal branches. Twelve weeks later, the IMA bypasses were selectively imaged by intra-arterial digital subtraction angiography and quantitatively evaluated. In all the animal experiments, the development of new vessels in the ischemic myocardium could be demonstrated angiographically. The formation of capillaries could also be demonstrated in humans and was found in all cases around the site of injection. A capillary network sprouting from the proximal part of the coronary artery could be shown to have bypassed the stenoses and rejoined the distal parts of the vessel.

Conclusions—We believe that the use of FGF-I for myocardial revascularization is in principle a new concept and that it may be particularly suitable for patients with additional peripheral stenoses that cannot be revascularized surgically. (*Circulation.* 1998;97:645–650.)

Key Words: growth substances ■ angiogenesis ■ coronary disease

For the cardiac surgeon who is attempting to treat CHD, the use of sections of autologous blood vessels as bypass material is subject to severe limitations. Autologous arterial conduits are in short supply, and segments of the saphenous vein do not remain patent for very long.[1,2] Furthermore, "complete" revascularization is limited if diffuse coronary arteriosclerosis is present and extensive, especially if there are additional peripheral stenoses.

In the search for alternative and/or additional treatment for improving the long-term prognosis, especially in diffuse CHD, attention has recently been directed toward natural angiogenesis.[3,9] Growth factors, especially FGF-I, have recently become of major importance because they can induce angiogenesis.[8,10–12]

Gimenez-Gallego et al[13] succeeded in elucidating the biochemical structure of FGF-I in 1985. Jaye et al[14] isolated human FGF-I from brain tissue in 1986. In 1991, Forough and coworkers[15] successfully used the technique of gene transfer to introduce the information for expressing human FGF-I into apathogenic *Escherichia coli*.

Our aim was to evaluate the information currently available on the biological effect of angiogenetic growth factors in animals and, if appropriate, to use human growth factor for the treatment of CHD. This involved (1) the production of human growth factor by genetic engineering, followed by its isolation, characterization, and purification; (2) using animal experiments to establish its angiogenetic potency and to exclude any possible pathogenic effect; and (3) using FGF-I clinically as an adjunct to coronary surgery and to demonstrate neoangiogenesis in the ischemic human myocardium.

Methods

Production and Purification of FGF-I

The production and purification of human FGF-I is a biochemically elaborate technique. The individual experimental steps have been reported elsewhere.[4,7]

Genetic engineering was used to produce human FGF-I from apathogenic strains of *E coli*, a plasmid containing the genetic information being introduced into the microorganisms.[15] These were kindly provided by Prof T. Maciag (Laboratory of Molecular Biology, American Red Cross, Rockville, Md). After production, FGF-I was eluted by heparin sepharose column chromatography, and several elution fractions were collected and purified by dialysis. Positive protein elution fractions were identified in the BIO-RAD assay[7] by SDS-PAGE,[16] and the biochemical isolation of FGF-I was confirmed by the Western blot method.[17] Further purification was obtained by HPLC.[18] The factors were lyophilized and stored at −32°C and diluted to 1 mL with NaCl solution containing 500 IU of heparin.

Chorioallantoic Membrane Assay

This established method, which provides a direct demonstration of the effect of growth factors on living tissue, was used to investigate the angiogenetic effect of FGF-1.[19,20] The growth of the allantoic systems can be directly observed by light microscopy. After incubation of 20 fertilized hen eggs for 13 days, the growth factor was applied to the membrane and covered with tissue culture coverslips. Four days later, the membrane was examined under the light microscope and directly compared with controls untreated with FGF-I or treated with heat-denatured FGF-I (70°C for 3 minutes).

Exclusion of the Pyrogenicity of FGF-I

Varying concentrations of FGF-I (0.01, 0.5, or 1.0 mg/kg body weight) were injected subcutaneously, intramuscularly, or intravenously into 27 New Zealand White rabbits, the solvent alone being used for an additional 13 controls. Thereafter, the rectal temperature was taken every half hour for 3 hours, hourly for the rest of the day, and every 8 hours for 12 days. A daily white cell count was also repeated for 12 days (see "Results"). In addition to this, the erythrocyte sedimentation rate and the C-reactive protein values were determined on the 3rd, 6th, 9th, and 12th days after the injection.

Confirmation of the Angiogenetic Potency of FGF-I in Animal Experiments

Supplementary to our earlier experiments,[6,7] the effect of FGF-I was also investigated in the ischemic hearts of inbred Lewis rats (a total of 275 animals, including 125 controls treated with heat-denatured FGF-I, 70°C for 3 minutes). The pericardium was opened via the abdominal wall and diaphragm, and two titanium clips were inserted at the apex of the left ventricle to induce myocardial ischemia. Growth factor (mean concentration of 10 μg) was then injected locally into the site. The coronary vessel system was imaged by aortic root angiography after 12 weeks and, finally, a specimen from the same myocardial region was evaluated histologically.

Clinical Use of FGF-I in Patients With CHD

This study was approved by the Medical Research Commission at the Phillips University of Marburg on August 10, 1993 (No. 47/93). This is the usual ethics commission for our hospital. Twenty patients without any history of infarction or cardiac surgery (14 men and 6 women; minimum age, 50 years) were subjected to an elective bypass operation for multivessel coronary heart disease. The growth factor was applied directly during the operation. As a control group, 20 patients who underwent the same procedure were given heat-denatured FGF-I (70°C for 3 minutes). The choice of treatment was completely random, the names being placed in sealed envelopes and selected in a blinded manner.

The details, nature, and aims of this procedure were explained beforehand to every patient who underwent the operation. In all cases, we received their fully informed consent. Both groups of patients were closely comparable with regard to clinical symptoms, accompanying disorders, cardiovascular risk factors, ventricular function, sex, and age. A comparable coronary morphology was found in both groups.

All patients had a further stenosis in the distal third of the LAD or at the origin of one of its branches in addition to a severe proximal stenosis. The mean ejection fraction of the left ventricle for all patients was 50%. The operative procedure for coronary revascularization with autologous grafts (an average per patient of 2 to 3 venous bypasses and 1 from the left IMA) was routinely performed. FGF-I (mean concen-

Figure 1. Intraoperative administration of growth factor.

tration, 0.01 mg/kg body weight) was injected into the myocardium, distal to the IMA/LAD anastomosis and close to the LAD, during the maintenance of the extracorporeal circulation and after completion of the distal anastomoses (Fig 1). In the control group, heat-denatured FGF-I was substituted for FGF-I. After 12 weeks, the IMA bypasses of all the patients were imaged selectively by transfemoral, intra-arterial, and digital subtraction angiography.

Angiograms obtained in this way were evaluated by means of EDP-assisted digital gray-value analysis, a universally recognized and well-established technique for demonstrating capillary neoangiogenesis.[21-26] Sites of interest both with and without FGF-I (meaning heat-denatured FGF-I) were selected in the vessels filled with contrast medium and in regions of the myocardium distal to the IMA/LAD anastomosis. One hundred pixels were selected from each site of interest and analyzed digitally. Complete blackening of the x-ray films was rated with a gray value of 150, and areas without blackening of the film were allotted a zero value. During the first 5 postoperative days, separate laboratory checks in addition to the routine postoperative follow-up procedures were made twice daily, and the temperature checked three times a day.

Results

After separation, purification, and stabilization, we were able to isolate human FGF-I in all 40 bacterial cultures and demonstrate its high degree of purity. Fig 2 shows an HPLC profile of the growth factor after routine purification. The peak values at the beginning and end of the profile represent impurities that could be identified as *E coli* proteins. FGF-I could be further separated by fractionated collection, and the control HPLC (Fig 3) merely shows the peak value of this fraction on an otherwise even baseline.
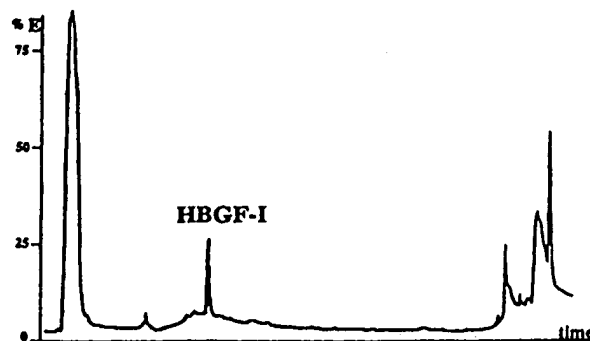

Figure 2. HPLC profile before high purification. HBGF-I indicates human FGF-I; %E, extinction.

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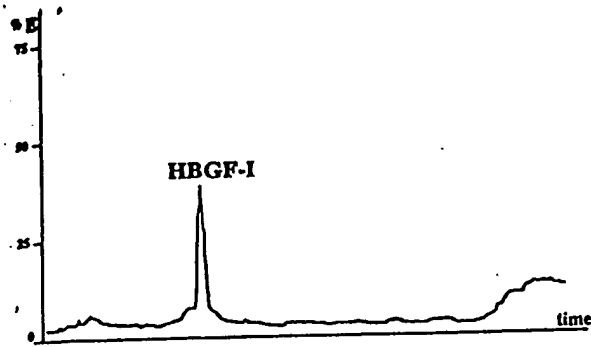C profile of
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Figure 3. HPLC profile after high purification. HBGF-I indicates human FGF-I; %E, extinction.

In the chorioallantoic membrane assay, the angiogenetic potency of FGF-I could be demonstrated in vivo. As early as 4 days after application of the factor, the vascular structure of the membrane was completely altered. Emanating radially from the site of application, an unequivocal growth of new vessels from the original host vessels had grown out into the periphery (Fig 4A). These structures were completely absent from the control group, and a normally developed reticular vascular pattern could be discerned (Fig 4B).

Pyrogenic effects of the human growth factor produced in this way could be definitively ruled out in the animal model. There was no significant rise of body temperature when checked at short intervals and no trace of an inflammatory reaction in comparison with the control group (n=13) in any of the 27 test animals during the period of observation. This result was independent of the concentration and the route of administration (intravenous, subcutaneous, or intramuscular) of the factor.

Earlier investigations into the application of FGF-I to the nonischemic rat heart made it possible to demonstrate neoangiogenesis both histologically and angiographically after 9 weeks in 11 of 12 test animals after the implantation of a tissue bridge pretreated with growth factor between the heart and thoracic aorta. In the control group without FGF-I (n=6), no signs of induced neoangiogenesis could be found.[4,7]

Unequivocal proof of induced neoangiogenesis was also found in the ischemic rat heart. In the test animals, in which myocardial ischemia had previously been induced with titanium clips and growth factor had subsequently been injected into the myocardium, a manifest accumulation of contrast medium was shown by aortic angiography at the site of the FGF-I injection 12 weeks later (Fig 5A), whereas such an accumulation of contrast medium did not appear in any of the control animals (Fig 5B). Histological examination of the myocardium revealed a threefold increase in the capillary density per square millimeter around the site of the FGF-I injection.

When the growth factor FGF-I was used clinically for the first time on the human heart, neoangiogenesis together with the development of a normal vascular appearance could be demonstrated angiographically, exactly as in the earlier animal experiments.[4,7] Selective imaging of the IMA bypasses by intra-arterial digital subtraction angiography confirmed the following result in all 20 patients: at the site of injection and in the distal areas supplied by the LAD, a pronounced accumulation of contrast

10 ng HBGF-I

without HBGF-I

Figure 4. A, Chorioallantoic membrane assay with application of the growth factor. B, Chorioallantoic membrane assay of the control group. HBGF-I indicates human FGF-I.

medium extended peripherally around the artery for ~3 to 4 cm, distal to the IMA/LAD anastomosis (Fig 6A). In the control angiograms of patients to whom only heat-denatured FGF-I had been given, the IMA/LAD anastomosis was also recognizable, but the accumulation of contrast medium described above was absent (Fig 6B). The angiograms of both the treated and control groups were recorded at a rate of four images per second, and these show

10 µgHBGF-1

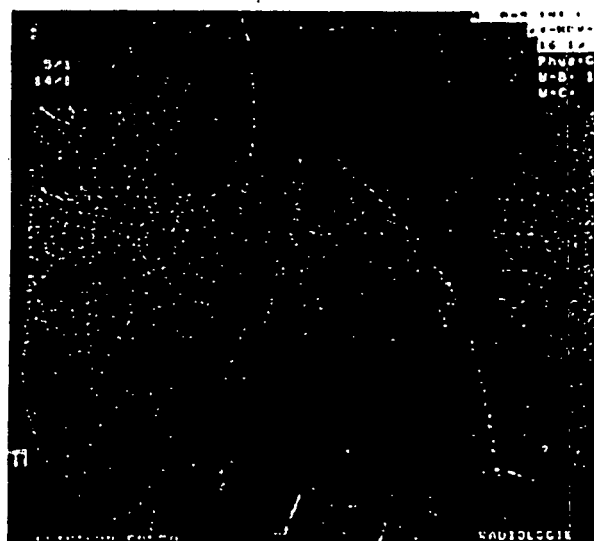10 µg/kg HBGF-1

without HBGF 1

Figure 5. A, Administration of the growth factor in ischemic rat heart with a clearly discernible accumulation of contrast medium at the site of injection. B, No discernible accumulation of contrast medium in the control group. HBGF-I indicates human FGF-I.

comparable distances between the beginning of the injection and visualization of the medium.

At the site of injection of the FGF-I, a capillary network could be seen sprouting out from the coronary artery into the myocardium. This enabled retrograde imaging of a stenosed diagonal branch to be performed (Fig 7A). Such "neocapillary vessels" can also provide a collateral circulation around additional distal stenoses of the LAD (Fig 7B) and bring about

without HBGF-I

Figure 6. A, Angiography after injection of the growth factor into the human heart shows a pronounced accumulation of contrast medium compared with the control group. B, Angiography in the control group does not show any increased accumulation of contrast medium around the IMA/LAD anastomosis. HBGF-I indicates human FGF-I.

retrograde filling of a short segment of the artery distal to the stenosis. In none of the angiograms of the treated patients taken 12 weeks after the operation were any new stenoses of the LAD detectable.

The results of EDP-assisted digital gray value analysis for quantification of the neoangiogenesis (Fig 8) gave a mean gray value of 124 for the vessels. The control myocardium reached

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Figure 8. Quantitative gray value analysis of contrast medium accumulation in the angiography shows a twofold to threefold increase in the local blood flow at the site of injection. HBGF-I indicates human FGF-I.

10 µg/kg HBGF-I

10 µg/kg HBGF-I

Figure 7. A, Collateralization of stenoses (arrow): a diagonal branch occluded just distal to its origin is filled through the newly grown capillaries. B, Collateralization of stenoses (arrow) by newly grown capillaries: the peripherally stenosed LAD is filled through these vessels. HBGF-I indicates human FGF-I.

conditions, and this naturally leads to the rapid growth of new capillaries and other blood vessels. Such a physiological process occurs in the development of the placenta, in fetal growth, and in wound healing, as well in the formation of collaterals in response to tissue ischemia. "Angiogenetic growth factors," which are biochemically polypeptides, are essential for such processes as capillary growth or neoangiogenesis. These growth factors (for instance, the human heparin-binding FGF-I) bring about their effect by significantly increasing cell proliferation, differentiation, and migration via a high-affinity receptor system on the surfaces of the endothelial cells.[8,10-12]

During the last few years, several working groups have been able to establish indications for the effective use of growth factors to improve blood flow in the presence of tissue ischemia in animal experiments.[3,9,27] Yanagisawa-Miwa et al[9] succeeded in demonstrating a significant collateralization together with reduction in the size of the infarct after intracoronary administration of growth factor in rabbits. Baffour et al[3] also observed a significant formation of collaterals in ischemic extremities after growth factor administration in animals. Albes et al[27] produced a distinct improvement in the blood flow in ischemic tracheal segments implanted subcutaneously in rabbits by injecting growth factor–enriched fibrin glue locally.

After growth factor was injected into the ischemic rat heart,[4,7] we were able to observe induced neoangiogenesis and confirm it angiographically. We were also able to prove histologically that this neoangiogenesis brings about the development of new vascular structures with a three-layered vessel wall. Angiographic imaging confirmed that these are anatomically normal capillaries and other blood vessels.

The production of human FGF-I by our molecular biological method has proved to be a complex but readily reproducible procedure. From the bacterial cultures, we are able to isolate the factor as a pure substance in sufficient quantities. By in vitro assay and as a result of extensive animal experiments, we were able to exclude the possible pyrogenic effects of FGF-I.

In earlier animal experiments,[4] we were able to demonstrate the proliferative and mitogenic effects of the growth factor on human saphenous vein endothelial cells. Endothelial cell cultures with added growth factor induced a confluent monolayer after only 5 to 9 days, whereas the monolayer was not complete before 7 to 11 days in the control group. In addition to determining the total cell count with a cell counter, we also confirmed this result by analyzing the rate of DNA synthesis by measuring the incorporation of ^{3}H-thymidine into the endothelial cell nuclei using the

a gray value of only 20, and that of the myocardium injected with FGF-I gave a value of 59 (Fig 8).

Discussion

Normal capillaries have a cell population with a low turnover rate of months or years. On occasion, however, a high turnover rate of this cell population is possible even under physiological

method of Klagsbrun and Shing.[28] The cell proliferative potency of FGF-I could be further intensified by adding heparin, a glycosaminoglycan protecting the growth factor from inactivation by cellular enzymes and from heat and chemical denaturation.[29]

On the basis of these in vitro and in vivo experiments, we established for the first time the efficacy of FGF-I for the treatment of CHD, and were able to demonstrate that it can induce neoangiogenesis in situ in the ischemic human heart. This possibility has been widely discussed for many years but never before attempted.

A dense capillary network appeared around the site of injection of the factor in the myocardium of all our treated patients. This capillary network is a true de novo vascular system. Emerging from the proximal segment of the LAD, it sprouts out into the surrounding myocardium, bringing about a twofold to threefold increase in the local blood supply through these newly formed functional vessels. We were able to use the recognized physiological effects of FGF-I (as they occur in the repair mechanism of wound healing or in collateralization of ischemic tissue) to induce neoangiogenesis in the human ischemic heart.

We also consider that administration of FGF-I (produced in this way by genetic engineering), combined with operative myocardial revascularization, may well be an especially appropriate treatment for patients with additional peripheral stenoses that cannot be treated surgically.

In our opinion, neoangiogenesis induced by FGF-I opens up new possibilities for the treatment of ischemic myocardial disease. Furthermore, it could become a new therapeutic concept in the management of diffuse CHD after alternative methods of administration have also been developed. This method of inducing neoangiogenesis is also conceivable as a therapeutic option in other regions of the cardiovascular system in which arterial occlusion has led to ischemia.[30] However, before any such possibilities are realized, many more clinical investigations will have to be performed.

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EXHIBIT C-3

Editorial

Angiogenic Therapy of the Human Heart

Judah Folkman, MD

The field of angiogenesis research was initiated 27 years ago by a hypothesis that tumors are angiogenesis-dependent.[1] Shortly thereafter, in the early 1970s, it became possible to passage vascular endothelial cells in vitro for the first time.[2] Bioassays for angiogenesis were developed subsequently throughout that decade. The early 1980s saw the purification of the first angiogenic factors.[3-6] By the mid-1980s, angiogenesis inhibitors began to be discovered.[7-9] Translation of these laboratory findings to clinical application started in 1989, when interferon alfa was first used for the treatment of life-threatening hemangiomas in infants.[10-12]

Clinical applications of angiogenesis research are being pursued along three general lines: (1) prognostic markers in cancer patients,[13,14] (2) antiangiogenic therapy (for review, see Reference 15), and (3) angiogenic therapy. The first angiogenic therapy of ischemic vascular disease was the administration of vascular endothelial growth factor (VEGF)/vascular permeability factor to patients with severe peripheral vascular disease in the lower limbs.[16]

In a landmark paper, Schumacher and colleagues now report the first angiogenic therapy of human coronary heart disease.[17] It is an important study, not only because the authors describe how they produced their own recombinant human fibroblast growth factor-1 (FGF-1, also called acidic fibroblast growth factor) and tested it in vitro and in vivo but also because they conducted a randomized controlled clinical trial. In 20 patients with three-vessel coronary artery disease who underwent two or three venous bypass grafts and one from the internal mammary artery, the angiogenic protein FGF-1 was injected into the myocardium close to the left anterior descending coronary artery and distal to its anastomosis with the internal mammary artery. FGF-1 was injected during extracorporeal surgery and again after completion of the anastomosis. Transfemoral, intra-arterial digital subtraction angiography 12 weeks later showed coronary artery neovascularization extending out from the area of FGF-1 injection. Stenoses distal to the anastomosis were bridged by neovascularization. This was similar to the neovascularization observed by the authors in rat hearts injected with FGF-1. Histological sections of rat myocardium showed a threefold increase in microvessel density. In 20 patients undergoing similar coronary artery bypass surgery in whom inactivated FGF-1 was injected, there was no evidence of myocardial neovascularization on the 12-week angiogram.

An advantage of this approach is that it induces local angiogenesis and appears to avoid high levels of circulating angiogenic activity that could possibly stimulate plaque angiogenesis and secondary plaque growth. Why does neovascularization persist for at least 12 weeks after only a single set of intramyocardial injections of the angiogenic protein? Perhaps persistent neovascularization was facilitated by upregulation of VEGF and its receptors in hypoxic tissue.[18] Furthermore, basic FGF and VEGF are synergistic mitogens for endothelial cells in vitro.[19,20] Also, FGF can increase expression of (or mobilize) VEGF.[21]

This report uses primarily anatomic studies to demonstrate increased myocardial neovascularization after angiogenic therapy. We look forward to the follow-up of these patients to learn whether they have significant functional improvement compared with the control group of patients who received inactive FGF. It may be difficult to discriminate the extent to which functional improvement is due to the angiogenic therapy per se, despite use of a control group, because of the concomitant internal mammary artery anastomosis and the relatively small number of patients in this study. Nevertheless, the angiographic documentation of myocardial revascularization suggests that functional improvement should follow.

Although major therapeutic advances in cardiology have been based on the general principles of control of blood pressure, regulation of cardiac rhythm, enhancement of myocardial contractile strength, increased diameter of narrowed coronary arteries, and lysis of intravascular thromboses, the report by Schumacher et al introduces a new modality, the regulation of blood vessel growth. If angiogenic therapy of the myocardium continues to live up to its potential as indicated by this report, we may witness novel refinements in future years as the molecular biology of endothelial cell and smooth cell growth is gradually uncovered. For example, the therapeutic induction of coronary arterial collaterals may someday be optimized by administration of appropriate mixtures of molecules that target different components of the vasculature, ie, the FGFs are mitogenic for vascular endothelial cells and smooth muscle, VEGF[22] is mitogenic primarily for endothelial cells, angiopoietin-1 mediates the recruitment of smooth muscle cells to the wall of new vessels,[23] and angiopoietin-2 appears to prevent or downregulate smooth muscle apposition to the walls of microvessels.[24] It is interesting that the methodology to discover these different vascular cell growth proteins emerged largely from investigations of mechanisms of tumor angiogenesis in studies funded primarily by the National Cancer Institute over many years. The report by Schumacher et al illustrates how unpredictable are the clinical applications that may arise from basic research in a different field.

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EXHIBIT C-4

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Growing New Blood Vessels with a Timed-Release Capsule of Growth Factor is a Promising Treatment for Heart Bypass Patients, Finds NHLBI Study

By The National Heart, Lung, and Blood Institute

Heart bypass patients treated with a timed-release capsule of a substance that promotes the growth of new blood vessels showed evidence of improved blood supply and heart function, according to a study supported by the National Heart, Lung, and Blood Institute (NHLBI) of the National Institutes of Health.

"Growing" blood vessels, a strategy called angiogenesis, is a promising experimental treatment for blocked arteries in bypass surgery patients for whom surgery alone would not adequately restore blood flow to the heart.

Dr. Michael Simons and colleagues at Harvard Medical School inserted timed-release capsules of basic fibroblast growth factor (bfGF) into the heart muscle of patients scheduled for bypass surgery. Patients received either a 10 microgram (mcg) or 100 mcg dose

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of the substance. Other patients
received a harmless placebo capsule
at the time of surgery. The relatively
small study (24 patients total) was
designed to test the safety and
effectiveness of the procedure.

The study, published in the
November 2, 1999 issue of
Circulation, found that there were
no serious adverse effects of the
treatment. Both magnetic resonance
imaging (MRI) and nuclear stress
testing were used to evaluate
changes in blood flow. Stress tests
showed a worsening of blood flow in
the placebo group, no change in the
10 mcg. group and significant
improvement in patients receiving
100 mcg. MRI results showed clear
improvement in blood flow in
patients given 100 mcg. Patients in
the highest dose group were free of
angina (chest pain) but some
patients in the placebo and low-dose
group experienced chest pain.

Simons and colleagues note that a
larger (Phase II) multi-center study
of this approach is currently
underway.

The National Heart, Lung, and Blood
Institute of The National Institutes of
Health. Press Release: **Growing New
Blood Vessels with a Timed-Release
Capsule of Growth Factor Is a
Promising Treatment for Heart Bypass
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(1 of 1)

United States Patent	5,652,225
Isner	July 29, 1997

Methods and products for nucleic acid delivery

Abstract

The present invention provides a method for the delivery of a nucleic acid to an arterial cell comprising contacting the cell with a hydrophilic polymer incorporating the nucleic acid. The nucleic acid may be any nucleic acid, including antisense DNA or RNA. The nucleic acid may encode hormones, enzymes, receptors or drugs of interest. The nucleic acid is selected based upon the desired therapeutic outcome. For example, in the treatment of ischemic diseases, one would select a DNA encoding an angiogenic protein. The nucleic acid may be carried by a microdelivery vehicle such as cationic liposomes and adenoviral vectors. DNA encoding different proteins may be used separately or simultaneously.

Inventors: **Isner; Jeffrey M. (Weston, MA)**
Assignee: **St. Elizabeth's Medical Center of Boston, Inc. (Boston, MA)**
Appl. No.: **675523**
Filed: **July 3, 1996**

U.S. Class:	514/44; 604/51; 604/52; 604/53; 536/23.5; 536/23.51; 435/320.1; 435/172.1; 435/172.3; 935/9; 935/22; 935/32; 935/33; 935/34; 935/52; 935/57; 424/93.2
Intern'l Class:	A01N 047/40
Field of Search:	514/44 604/51,52,53 536/23.5,23.51 435/320.1,172.1,172.3,235.1,240.2 935/9,22,32,33,34,52,57 424/93.2

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Primary Examiner: Low; Christopher S. F.
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Parent Case Text

This is a continuation of application Ser. No. 08/318,045 filed on Oct. 4, 1994 now abandoned.

Claims

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1. A method for inducing the formation of new blood vessels in a desired target tissue in a human host, comprising contacting an arterial cell in an artery or blood vessel via a balloon catheter coated with a hydrogel polymer admixed with a first DNA encoding an angiogenic protein selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor .alpha. and .beta., platelet-derived endothelial

growth factor, platelet-derived growth factor, tumor necrosis factor .alpha., hepatocyte growth factor and insulin like growth factor and having an operably linked secretory signal sequence or a first DNA encoding a modified angiogenic protein selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor .alpha. and .beta., platelet-derived endothelial growth factor, platelet-derived growth factor, tumor necrosis factor .alpha., hepatocyte growth factor and insulin like growth factor having an operably linked secretory signal sequence, wherein said anglogenic protein induces new blood vessel formation when expressed in said target tissue in an amount effective to induce new blood vessel formation.

2. The method of claim 1, wherein the angiogenic protein is vascular endothelial growth factor.

3. The method of claim 1, wherein the hydrogel polymer is selected from the group consisting of polycarboxylic acids, cellulosic polymers, gelatin, polyvinylpyrrolidone, maleic anhydride polymers, polyamides, polyvinyl alcohols, and polyethylene oxides.

4. The method of claim 1, wherein the hydrogel polymer is a polyacrylic acid polymer.

5. The method of claim 1, wherein the hydrogel polymer is admixed with a second DNA encoding an angiogenic protein selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor .alpha. and .beta., platelet-derived endothelial growth factor, platelet-derived growth factor, tumor necrosis factor .alpha., hepatocyte growth factor and insulin like growth factor and having an operably linked secretory signal sequence or a second DNA encoding a modified angiogenic protein selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor .alpha. and .beta., platelet-derived endothelial growth factor, platelet-derived growth factor, tumor necrosis factor .alpha., hepatocyte growth factor and insulin like growth factor having an operably linked secretory signal sequence, wherein said angiogenic protein induces new blood vessel formation when expressed in said target tissue in an amount effective to induce new blood vessel formation, and wherein said second DNA is not the same as said first DNA.

6. A method for inducing the formation of new blood vessels in a desired target tissue in a human host, comprising contacting an arterial cell in an artery or blood vessel via a balloon catheter coated with a hydrogel polymer admixed with DNA encoding vascular endothelial growth factor and which is expressed in an amount effective to induce new blood vessel formation.

Description

FIELD OF THE INVENTION

The present invention relates to delivery of nucleic acid to arterial cells and compositions therefor.

BACKGROUND OF THE INVENTION

Work from several laboratories (Nabel, et al., Science, 249:1285-1288 (1990); Lim, et al., Circulation, 83:2007-2011 (1991); Flugelman, et al., Circulation, 85:1110-1117 (1992); Leclerc, et al., J. Clin. Invest., 90:936-944 (1992); Chapman, et al., Circ. Res., 71: 27-33 (1992); Riessen, et al., Hum. Gene Ther., 4: 749-758 (1993); and Takeshita, et al., J. Clin. Invest., 93:652-661 (1994), has demonstrated

that recombinant marker genes could be transferred to the vasculature of live animals.

Gene delivery systems employed to date have been characterized by two principal components: a macrodelivery device designed to deliver the DNA/carrier mixture to the appropriate segment of the vessel, and microdelivery vehicles, such as liposomes, utilized to promote transmembrane entry of DNA into the cells of the arterial wall. Macrodelivery has typically been achieved using one of two catheters initially developed for local drug delivery: a double-balloon catheter, intended to localize a serum-free arterial segment into which the carrier/DNA mixture can be injected, or a porous-balloon catheter, designed to inject gene solutions into the arterial wall under pressure. Jorgensen et al., Lancet 1:1106-1108, (1989); Wolinsky, et al., J. Am. Coil. Cardiol., 15:475-485 (1990); March et al., Cardio Intervention, 2:11-26 (1992)); WO93/00051 and WO93/00052.

Double balloon catheters are catheters which have balloons which, when inflated within an artery, leave a space between the balloons. The prior efforts have involved infusing DNA-containing material between the balloons, allowing the DNA material to sit for a period of time to allow transfer to the cells, and then deflating the balloons, allowing the remaining genetic material to flush down the artery. Perforated balloons are balloons which have small holes in them, typically formed by lasers. In use, fluid containing the genetic material is expelled through the holes in the balloons and into contact with the endothelial cells in the artery. These gene delivery systems however, have been compromised by issues relating to efficacy and/or safety.

Certain liabilities, however, inherent in the use of double-balloon and porous balloon catheters have been identified. For example, neither double-balloon nor porous balloon catheters can be used to perform the angioplasty itself. Thus, in those applications requiring both angioplasty and drug delivery, e.g., to inhibit restenosis, two procedures must be preformed. Additionally, the double balloon typically requires long incubation times of 20-30 min., while the high-velocity jets responsible for transmural drug delivery from the porous balloon catheter have been associated with arterial perforation and/or extensive inflammatory infiltration (Wolinsky, et al., J. Am. Coil. Cardiol., 15:475-481 (1990).

SUMMARY OF THE INVENTION

It has now been discovered that nucleic acids can be delivered to cells of an artery or blood vessel by contacting the cells with a hydrophilic polymer incorporating the nucleic acid, thus avoiding the use of a double-balloon or porous balloon catheter and the problems associated with such delivery systems. It has also been demonstrated that, unexpectedly, the percentage of transduced arterial cells is significantly higher using the present invention compared with use of a double-balloon catheter.

By "arterial cells" is meant the cells commonly found in mammalian arteries, including endothelial cells, smooth muscle cells, connective tissue cells and other cells commonly found in the arterial structure.

By "nucleic acid" is meant DNA and RNA, including antisense DNA or RNA.

It has further been discovered that a DNA encoding an angiogenic protein (a protein capable of inducing angiogenesis, i.e., the formation of new blood vessels), delivered by the method of the present invention is expressed by the arterial cell and induces angiogenesis in tissues perfused by the treated blood vessels. This allows for the treatment of diseases associated with vascular occlusion in a variety of target tissues, such as limb ischemia, ischemic cardiomyopathy, myocardial ischemia, cerebral ischemia and portal hypertension.

The present invention provides a method for the delivery of a nucleic acid to an arterial cell comprising contacting the cell with a hydrophilic polymer incorporating the nucleic acid. The nucleic acid may be any nucleic acid, DNA and RNA, including antisense DNA or RNA. The DNA may encode hormones, enzymes, receptors or drugs of interest. The nucleic acid is selected based upon the desired therapeutic outcome. For example, in the treatment of ischemic diseases, the genetic material of choice is DNA encoding an angiogenic protein. The nucleic acid may be carried by a microdelivery vehicle such as cationic liposomes and adenoviral vectors. DNA encoding different proteins may be used separately or simultaneously.

The hydrophilic polymer is selected to allow incorporation of the nucleic acid to be delivered to the arterial cell and its release when the hydrophilic polymer contacts the arterial cell. Preferably, the hydrophilic polymer is a hydrogel polymer. Other hydrophilic polymers will work, so long as they can retain the genetic material of the present invention, so that, on contact with arterial cells, transfer of genetic material occurs.

Suitable hydrogel polymers include, for example, those selected from the group consisting of polycarboxylic acids, cellulosic polymers, gelatin, polyvinylpyrrolidone, maleic anhydride polymers, polyamides, polyvinyl alcohols, and polyethylene oxides. The hydrogel polymer is preferably polyacrylic acid.

Without wishing to be bound by theory, one reason that the use of hydrogel, and particularly with hydrogel coated balloon catheters, is believed to provide improved results over, for example, prior treatments with double balloon catheters, is that the use of standard balloon catheters with hydrogel surfaces causes the hydrogel not only to contact the endothelial cells which line the interior of the arteries, but also displaces the endothelial cells sufficiently to permit contact between the hydrogel and the smooth muscle cells which underlie the endothelial cell layer. This permits expression of polypeptides in different arterial cell types, which enhances the kinds and amounts of therapeutic polypeptides which can be produced in accordance with this invention. For example, as indicated in the examples below, the present method successfully produces sufficient amounts of vascular endothelial growth factor (VEGF) to cause angiogenesis downstream from a DNA/arterial contact point, despite the fact that VEGF is not normally produced even by transformed endothelial cells, but is produced by smooth muscle cells of the type that surround the endothelial cells in the artery.

The arterial cell may be contacted with the hydrophilic polymer incorporating the DNA by means of an applicator such as a catheter which is coated with the DNA-bearing hydrophilic polymer. Preferably, the applicator can exert some pressure against the arterial cells, to improve contact between the nucleic acid-bearing hydrophilic polymer and the arterial cells. Thus a balloon catheter is preferred. Preferably, the hydrophilic polymer coats at least a portion of an inflatable balloon of the balloon catheter.

The present invention further includes compositions comprising hydrophilic polymers incorporating nucleic acid. Preferably the hydrophilic polymer is a hydrogel and the nucleic acid is DNA which encodes an angiogenic protein.

The present invention also provides kits for application of genetic material to the interior of an artery or similar bodily cavity, comprising a substrate, such as a catheter or a suitably shaped rod, and a source of genetic material comprising the DNA coding for the desired therapeutic polypeptide. Preferably, the present invention is directed to a catheter adapted for insertion into a blood vessel, having a balloon element adapted to be inserted into the vessel and expandable against the walls of the

vessel. At least a portion of the balloon element is defined by a coating of a hydrophilic polymer, and incorporated within the hydrophilic polymer coating, a nucleic acid to be delivered to the arterial cell. The hydrophilic polymer is preferably a hydrogel polymer, most preferably a hydrophilic polyacrylic acid polymer.

The present invention also provides a method for inducing angiogenesis in a desired target tissue, comprising delivering a DNA encoding an angiogenic protein to an arterial cell in an artery or blood vessel perfusing the target tissue.

Other aspects of the invention are discussed infra.

As used herein the term "angiogenic protein" means any protein, polypeptide, mutein or portion thereof that is capable of inducing the formation of new blood vessels. Such proteins include, for example, acidic and basic fibroblast growth factors (aFGF and bFGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor .alpha. and .beta. (TGF-.alpha. and TFG-.beta.), platelet-derived endothelial growth factor (PD-ECGF), platelet-derived growth factor (PDGF), tumor necrosis factor .alpha. (TNF-.alpha.), hepatocyte growth factor (HGF) and insulin like growth factor. Preferably, the angiogenic protein contains a secretory signal sequence allowing for secretion of the protein from the arterial cell. VEGF is a preferred protein.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1(a) and 1(b) show the rabbit ischemic hindlimb model. FIG. 1(a)is a representative angiogram recorded 10 days after surgery. Once the femoral artery is excised, thrombotic occlusion of the external lilac artery extends retrograde to its origin from the common lilac (arrow). Open arrow indicates the site of arterial gene transfer. In FIG. 1(b) the shaded segment of femoral artery has been excised.

FIGS. 2(a), 2(b) and 2(c) illustrate (a) RT-PCR analysis of transfected arteries, (b) Southern blot analysis of RT-PCR products and (c) nucleotide sequence of the RT-PCR product from transfected rabbit lilac artery. In FIGS. 2(a) and 2(b) the expression of the human VEGF mRNA was evident in the rabbit lilac artery (lane 4) and cultured rabbit vascular smooth muscle cells (lane 6, positive control) which were transfected with human VEGF gene. Arrows indicate position of VEGF band at 258 bp. Lane 1 depicts the results using a molecular weight marker, namely pGEM3zf(-) digested with Hae III; lane 2 is a negative control (no RNA); lane 3 is a second negative control (rabbit lilac artery transfected with .beta.-galactosidase expression plasmid); and lane 5 is a further negative control (PCR analysis of the VEGF-transfected lilac artery excluding the reverse transcriptase reaction). FIG. 2(c) shows the nucleotide sequence of the RT-PCR product from a transfected rabbit lilac artery. Direct sequencing of the 258 bp bands obtained by RT-PCR confirmed that this band represented the human VEGF sequence. The sequence designated in 2(c) corresponds to amino acids 69 to 75 of the VEGF peptide. Asterisks denote the nucleotides which are not conserved among different species of the VEGF gene (rat, mouse, bovine, guinea pig) demonstrating that the exogenous human gene was amplified by the RT-PCR procedure.

FIGS. 3A, 3B, 3C, 3D, 3E and 3F comprise internal lilac angiography of a control rabbit at (A) day 0 (pre-transfection), (B) day 10, and (C) day 30 post-transfection, and of a VEGF-transfected rabbit at (D) day 0, (E) day 10, and (F) day 30 post-transfection. In contrast to the control, angiographic examination of the VEGF-transfected animal discloses extensive collateral artery formation.

FIGS. 4(a), 4(b) and 4(c) are graphs illustrating the effect of VEGF-transfection on revascularization

in an ischemic limb model. FIG. 4(a) the angiographic score at day 0 (immediately prior to transfection), and days 10 and 30 post-transfection. FIG. 4(b) Calf Blood pressure ratio at day 0, and at days 10 and 30 post-transfection. FIG. 4(c) depicts capillary density at day 30 post-transfection. (*p<0.05,**p<0.01)

FIGS. 5(a) and 5(b) illustrate alkaline phosphatase staining of ischemic hindlimb muscle, counterstained with eosin. FIG. 5(a) depicts the muscle of an animal transfected with pGSVLacZ. FIG. 5(b) depicts the muscle of an animal transfected with phVEGF.sub.165. The dark staining indicates capillaries as shown by the arrows.

FIG. 6 illustrates a diagrammatical cross section of a balloon catheter having a hydrophilic surface bearing genetic material in accordance with the present invention, in place within an artery.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for the delivery of nucleic acid to an arterial cell comprising contacting the cell with a hydrophilic polymer incorporating the nucleic acid.

The nucleic acid may be any nucleic acid which when introduced to the arterial cells provides a therapeutic effect. The nucleic acid is selected based upon the desired therapeutic outcome. For example, in the treatment of ischemic diseases, one genetic material of choice would be a DNA encoding an angiogenic protein. DNA useful in the present invention include those that encode hormones, enzymes, receptors or drugs of interest. The DNA can include genes encoding polypeptides either absent, produced in diminished quantities, or produced in mutant form in individuals suffering from a genetic disease. Additionally it is of interest to use DNA encoding polypeptides for secretion from the target cell so as to provide for a systemic effect by the protein encoded by the DNA. Specific DNA's of interest include those encoding hemoglobin, interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-4, interleukin-5, interleukin-6, interleukin-7, interleukin-8, interleukin-9, interleukin-10, interleukin-11, etc., GM-CSF, G-CSF, M-CSR, human growth factor, insulin, factor VIII, factor IX, tPA, LDL receptors, tumor necrosis factor, PDGF, EGF, NGF, IL-1ra, EPO, .beta.-globin and the like, as well as biologically active muteins of these proteins. The nucleic acid utilized may also be "anti-sense" DNA or RNA, which binds to DNA or RNA and blocks the production of harmful molecules. In addition, the DNA carried to the arterial cells in accordance with the present invention may code for polypeptides which prevent the replication of harmful viruses or block the production of smooth muscle cells in arterial walls to prevent restenosis.

Antisense RNA molecules are known to be useful for regulating translation within the cell. Antisense RNA molecules can be produced from the corresponding gene sequences. The antisense molecules can be used as a therapeutic to regulate gene expression associated with a particular disease.

The antisense molecules are obtained from a nucleotide sequence by reversing the orientation of the coding region with regard to the promoter. Thus, the antisense RNA is complementary to the corresponding mRNA. For a review of antisense design see Green, et al., Ann. Rev. Biochem. 55:569-597 (1986), which is hereby incorporated by reference. The antisense sequences can contain modified sugar phosphate backbones to increase stability and make them less sensitive to RNase activity. Examples of the modifications are described by Rossi, et al., Pharmacol, Ther. 50(2):245-354, (1991).

In certain therapeutic applications, such as in the treatment of ischemic diseases, it may be desirable to induce angiogenesis, i.e., the formation of new blood vessels. For such applications, DNA's encoding growth factors, polypeptides or proteins, capable of inducing angiogenesis are selected. Folkman, et

al., Science, 235:442-447 (1987). These include, for example, acidic and basic fibroblast growth factors (aFGF and bFGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor .alpha. and .beta. (TGF-.beta. and TGF-.beta.), platelet-derived endothelial cell growth factor (PD-ECGF), platelet-derived growth factor (PDGF) itself, tumor necrosis factor .alpha. (TNF-.alpha.), hepatocyte growth factor (HGF) and insulin like growth factor. See, Klagsbrun, et al., Annu. Rev. Physiol., 53:217-239 (1991) and Folkman, et al., J. Biol. Chem. 267:10931-10934 (1992). Muteins or fragments of an angiogenic protein may be used as long as they induce or promote the formation of new blood vessels.

Recent investigations have established the feasibility of using recombinant formulations of such angiogenic growth factors to expedite and/or augment collateral artery development in animal models of myocardial and hindlimb ischemia. See, Baffour, et al., J. Vasc. Surg., 16:181-191 (1992) (bFGF); Pu, et al, Circulation, 88:208-215 (1993) (aFGF); Yanagisawa-Miwa, et al., Science, 257:1401-1403 (1992) (bFGF); Ferrara, et al., Biochem. Biophys. Res. Commun., 161:851-855 (1989) (VEGF).

VEGF was also purified independently as a tumor-secreted factor that included vascular permeability by the Miles assay (Keck, et al, Science, 246:1309-1342 (1989) and Connolly, et al., J. Biol Chem., 264:20017-20024 (1989)), and thus its alternate designation, vascular permeability factor (VPF). VEGF is a preferred angiogenic protein. Two features distinguish VEGF from other heparin-binding, angiogenic growth factors. First, the NH.sub.2 terminus of VEGF is preceded by a typical signal sequence; therefore, unlike bFGF, VEGF can be secreted by intact cells. Second, its high-affinity binding sites, shown to include the tyrosine kinase receptors Flt-1 and Flt-1/KDR are present on endothelial cells. Ferrara, et al., Biochem. Biophys. Res. Commun., 161:851-855 (1989) and Conn, et al., Proc. Natl. Acad. Sci. USA, 87:1323-1327 (1990). (Interaction of VEGF with lower affinity binding sites has been shown to induce mononuclear phagocyte chemotaxis). Shen, et al., Blood, 81:2767-2773 (1993) and Clauss, et al., J. Exp. Med., 172:1535-1545 (1990).

Evidence that VEGF stimulates angiogenesis in vivo had been developed in experiments performed on rat and rabbit cornea (Levy, et al., Growth Factors, 2:9-19 (1989) and Connolly, et al., J. Clin. Invest., 84:1470-1478 (1989)), the chorioallantoic membrane (Ferrara, et al., Biochem Biophys Res Commun., 161:851-855 (1989)), and the rabbit bone graft model. Connolly, et al., J. Clin. Invest., 84:1470-1478 (1989).

Preferably, the angiogenic protein contains a secretory signal sequence that facilitates secretion of the protein from the arterial cell. Angiogenic proteins having native signal sequences, e.g., VEGF, are preferred. Angiogenic proteins that do not have native signal sequences, e.g., bFGF, can be modified to contain such sequences using routine genetic manipulation techniques. See, Nabel et al., Nature 362:844 (1993).

The nucleotide sequence of numerous peptides and proteins, including angiogenic proteins, are readily available through a number of computer data bases, for example, GenBank, EMBL and Swiss-Prot. Using this information, a DNA segment encoding the desired may be chemically synthesized or, alternatively, such a DNA segment may be obtained using routine procedures in the art, e.g, PCR amplification.

To simplify the manipulation and handling of the DNA, prior to introduction to the arterial cell, the DNA is preferably inserted into a vector, e.g., a plasmid vector such as pUC118, pBR322, or other known plasmid vectors, that includes, for example, an E. Coli origin of replication. See, Sambrook, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory press, (1989). The plasmid vector may also include a selectable marker such as the .beta.-lactamase gene for ampicillin

resistance, provided that the marker polypeptide does not adversely effect the metabolism of the organism being treated. Additionally, if necessary, the DNA may be operably linked to a promoter/enhancer region capable of driving expression of the protein in the arterial cell. An example of a suitable promoter is the 763-base-pair cytomegalovirus (CMV) promoter. Normally, an enhancer is not necessary when the CMV promoter is used. The RSV and MMT promoters may also be used. Certain proteins can expressed using their native promoter.

If desired, the DNA may be used with a microdelivery vehicle such as cationic liposomes and adenoviral vectors. For a review of the procedures for liposome preparation, targeting and delivery of contents, see Mannino and Gould-Fogerite, Bio Techniques, 6:682 (1988). See also, Felgner and Holm, Bethesda Res. Lab. Focus, 11(2):21 (1989) and Maurer, R. A., Bethesda Res. Lab. Focus, 11 (2):25 (1989). Replication-defective recombinant adenoviral vectors, can be produced in accordance with known techniques. See, Quantin, et al., Proc. Natl. Acad. Sci. USA, 89:2581-2584 (1992); Stratford-Perricadet, et al., J. Clin. Invest., 90:626-630 (1992); and Rosenfeld, et al., Cell, 68:143-155 (1992).

In certain situations, it may be desirable to use DNA's encoding two or more different proteins in order optimize the therapeutic outcome. For example, DNA encoding two angiogenic proteins, e.g., VEGF and bFGF, can be used, and provides an improvement over the use of bFGF alone. Or an angiogenic factor can be combined with other genes or their encoded gene products to enhance the activity of targeted cells, while simultaneously inducing angiogenesis, including, for example, nitric oxide synthase, L-argine, fibronectin, urokinase, plasminogen activator and heparin.

The hydrophilic polymer is selected to allow incorporation of the DNA to be delivered to the arterial cell and its release when the hydrophilic polymer contacts the arterial cell.

Preferably, the hydrophilic polymer is a hydrogel polymer, a cross-linked polymer material formed from the combination of a colloid and water. Cross-linking reduces solubility and produces a jelly-like polymer that is characterized by the ability to swell and absorb liquid, e.g., that containing the DNA. Suitable hydrogel polymers include, for example, those selected from the group consisting of polycarboxylic acids, cellulosic polymers, gelatin, polyvinylpyrrolidone, maleic anhydride polymers, polyamides, polyvinyl alcohols, and polyethylene oxides. Preferred hydrogels are polyacrylic acid polymers available as HYDROPLUS (Mansfield Boston Scientific Corp., Watertown, Mass.) and described in U.S. Pat. No. 5,091,205.

The nucleic acid in aqueous solution is incorporated into the hydrophilic polymer to form a nucleic acid-hydrophilic polymer composition. The nucleic acid is incorporated without complexing or chemical reaction with the hydrophilic polymer, and is preferably relatively freely released therefrom when placed in contact with the arterial cells. The resulting structure comprises a support, e.g. the balloon of the balloon catheter, on which is mounted the hydrogel, in or on which is incorporated the desired DNA and its associated vehicle, e.g., phage or plasmid vector. The hydrophilic polymer is preferably adhered to the support, so that after application of the DNA to the target cells, the hydrophilic polymer is removed with the support.

An arterial cell is contacted with the nucleic acid-hydrophilic polymer composition by any means familiar to the skilled artisan. The preferred means is a balloon catheter having the hydrophilic polymer on its outer surface, which permits the contact between the hydrophilic polymer bearing the nucleic acid to be transferred and the arterial cells to be made with some pressure, thus facilitating the transfer of the nucleic acid to the cells. However, other supports for the hydrophilic polymer are also useful, such as catheters or solid rods having a surface of hydrophilic polymer. Preferably, the catheters or

rods or other substrates which are flexible, to facilitate threading through the arteries to reach the point of intended application. For cells that are not in tubular arteries, other types of catheters, rods or needles may be used.

When a hydrophillic arterial balloon is used, it is not necessary to protect the balloon prior to inflation, since relatively little of the nucleic acid is lost in transit to the treatment site until the balloon is inflated and the hydrophilic polymer bearing the nucleic acid is pressed against the arterial cells. When hydrophilic polymer-surfaced catheters or rods are used as the vehicle or substrate, the surface can protected, e.g. by a sheath, until the point of intended application is reached, and then the protection removed to permit the hydrophilic polymer bearing the nucleic acid to contact the arterial cells.

The vehicle, be it arterial balloon, catheter, flexible rod or other shaped vehicle, can be furnished with means to assist in accurate placement within the intended body cavity. For example, it can be furnished with a radioactive element, or made radio-opaque, furnished with means permitting easy location using ultrasound, etc.

Preferably, the nucleic acid-hydrophilic composition contacts the arterial cell by means of a catheter. The catheter is preferably a balloon catheter constructed for insertion in a blood vessel and has a catheter shaft and an expandable dilation balloon mounted on the catheter shaft. At least a portion of the exterior surface of the expandable portion is defined by a coating of a tenaciously adhered hydrophilic. Incorporated in the hydrophilic polymer is an aqueous solution of the DNA to be delivered to the arterial cells.

In general, when dry, the hydrophilic polymer (preferably hydrogel) coating is preferably on the order of about 1 to 10 microns thick, with a 2 to 5 micron coating typical. Very thin hydrogel coatings, e.g., of about 0.2-0.3 microns (dry) and much thicker hydrogel coatings, e.g., more than 10 microns (dry), are also possible. Typically, hydrogel coating thickness may swell by about a factor of 2 to 10 or more when the hydrogel coating is hydrated.

Procedures for preparing a balloon with a hydrogel coating are set forth in U.S. Pat. No. 5,304,121, the disclosure of which is incorporated herein by reference.

A representative catheter is set forth in FIG. 6. Referring to FIG. 6, 1 is the wall of the blood vessel. The figure shows the catheter body 2 held in place by the inflation of an inflation balloon 3. The balloon comprises a hydrogel coating 4 incorporating DNA 5.

In use, the DNA, for example, is applied ex vivo to the hydrophilic polymer coating of the balloon. To facilitate application, the balloon may be inflated. If necessary, the polymer may be dried with warm air and the DNA application repeated. The amount of DNA to be applied to the arterial surface depends on the purpose of the DNA and the ability of the DNA to be expressed in the arterial cells. Generally, the amount of naked DNA applied to the balloon catheter is between about 0.1 and 100 .mu.g/mm.sup.2, more preferably between about 0.5 and about 20 .mu.g/mm.sup.2, most preferably between about 1.5 and about 8 .mu.g/mm.sup.2. Preferably, between 0.5 mg and 5 mg of DNA are applied to the hydrogel coating of a balloon catheter having an inflated lateral area of about 630 mm.sup.2 (e.g., a balloon catheter having an inflated diameter of about 5 mm and a length of about 40 mm), providing a surface having about 0.8 to about 8 .mu.g/mm.sup.2 of DNA when the balloon is inflated and contacts the interior of the artery. More preferably, between 1 mg and 3 mg of DNA are applied to the polymer, providing a DNA loading of about 1.6 to about 4.8 .mu.g/mm.sup.2.

The catheter is inserted using standard percutaneous application techniques and directed to the desired location, e.g., an artery perfusing the target tissue. For example, in the treatment of patients with occlusive peripheral arterial disease (PAD), the balloon is directed towards an artery of the leg, e.g., lilac. Once the balloon has reached its desired location, it is inflated such that the hydrogel coating of the balloon contacts the arterial cells located on the walls of the artery and remains inflated for a time sufficient to allow transfer of the DNA encoding the angiogenic protein from the hydrogel to the arterial cells. Preferred periods of balloon inflation range from 30 seconds to 30 minutes, more preferably 1 minute to 5 minutes. Surprisingly, that is normally sufficient time to permit transfer of the DNA by the method of the present invention.

Once transferred, the DNA coding for the desired therapeutic polypeptide is expressed by the arterial cells for a period of time sufficient for treatment of the condition of interest. Because the vectors containing the DNA of interest are not normally incorporated into the genome of the cells, however, expression of the protein of interest takes place for only a limited time. Typically, the therapeutic protein is only expressed in therapeutic levels for about two days to several weeks, preferably for about 1-2 weeks. Reapplication of the DNA can be utilized to provide additional periods of expression of the therapeutic polypeptide. If desired, use of a retrovirus vector to incorporate the heterologous DNA into the genome of the arterial cells will increase the length of time during which the therapeutic polypeptide is expressed, from several weeks to indefinitely.

In one preferred application, the DNA-hydrogel polymer composition can be used to deliver a DNA encoding an angiogenic protein to an arterial cell in an artery or blood vessel perfusing the target tissue. Expression of the angiogenic protein and its secretion from the arterial cell induces angiogenesis, i.e., the formation of new blood vessels, in target tissues perfused by the artery or blood vessels, allowing for the treatment of diseases associated with vascular occlusion such as limb ischemia, ischemic cardiomyopathy, myocardial ischemia, cerebral ischemia and portal hypertension.

The present invention makes genetic treatment possible which can correct heretofore incorrectable problems.

The present invention is further illustrated by the following examples. These examples are provided to aid in the understanding of the invention and are not construed as a limitation thereof.

EXAMPLE 1

Direct Gene Transfer with Hydrogel Polymer Balloon Catheter Applied to an Angioplasty Catheter Balloon Can be Used to Effect Direct Gene Transfer to the Arterial Wall.

DNA solution was applied to the surface of an angioplasty catheter balloon with a hydrogel polymer (marketed under the mark Slider.TM. with Hydroplus.RTM. by Mansfield Boston Scientific Corp., Watertown, Mass.). The catheter was constructed with a single polyethylene balloon, 2.0 mm in diameter and 2.0 cm in length. The Hydroplus.RTM. coating consists of a hydrophilic polyacrylic acid polymer, crosslinked via an isocyanate onto the balloon to form an ultra-high molecular weight hydrogel with tight adherence to the balloon surface. The thickness of the hydrogel coating when dry measures between 3-5 .mu.m; upon exposure to an aqueous environment, the coating swells to 2-3 times its initially dry thickness. In order to apply DNA to the catheter, the balloon was inflated to 4 atm, following which 20 .mu.l of DNA solution were pipetted and distributed onto the balloon surface using a sterile pipette tip. After the balloon's hydrogel polymer was covered with a homogeneous film of DNA solution, the hydrogel was dried with warm air. The above procedure was then repeated, resulting in a total of 40 .mu.l of DNA solution applied to the balloon.

For percutaneous application, luciferase DNA concentration was 3.27 .mu.g/.mu.l. DNA was dissolved in TE buffer (10 mM Tris, 1 mM EDTA).

(Attempts were made to apply DNA solution to standard uncoated balloons as well. The hydrophobic surface of the polyethylene balloon, however, made it impossible to cover the balloon with a film of DNA solution.)

To determine the total amount of DNA which is successfully absorbed onto the balloon surface, 5 hydrogel balloons were coated with 40 .mu.l DNA (2 .mu.g DNA/.mu.l) containing a small amount of .sup.35 S-labeled luciferase plasmid. (Levy, et al., Growth Factors, 2:1535-1545 (1990)). A random primed DNA labeling kit (United States Biochemical, Cleveland, Ohio) was used for the labeling reaction an unincorporated nucleotides were remove by ethanol precipitation. After the coating procedure, the catheter tip was placed in 0.5 ml water for 15 minutes at room temperature, and 1.0 ml gel solubilizer (Solvable,.TM. New England Nuclear, Boston, Mass.) for 3 hours at 50.degree. C. to dissolve the gel before the scintillation fluid was added. The amount of DNA on the balloon was calculated from the quotient: [counts per minute (cpm) in a scintillation vial containing the balloon]/[cpm in a vial containing 40 .mu.l of the same lot of labeled DNA (80 .mu.g)]. Scintillation counts were corrected for quench and chemiluminescence.

After coating hydrogel balloons with 40 .mu.l of DNA solution (containing 80 .mu.g of radiolabeled DNA), and drying the gel, the magnitude of DNA retained on the hydrogel balloon was determined by comparing the amount of radioactivity on the balloons to the amount of radioactivity in 40 .mu.l of the original radiolabeled DNA solution. Scintillation counting revealed that 97.+-.2% (n=5) of the radioactively labeled DNA remained on the hydrogel coated balloon, corresponding to 78.+-.1.5 .mu.g of luciferase DNA.

Reporter Genes

The firefly luciferase gene and the gene for nuclear-specific .beta.-galactosidase (.beta.-gal) were used as reporter genes to monitor the results of the transfection procedures. The luciferase expression vector, pRSVLUC (courtesy of Dr. Allen Brasier, Massachusetts General Hospital, Boston, Mass.), consist of a full length Photinus pyralis luciferase cDNA (pJD 204) (de Wet et al., 1987) inserted into a PGEM3-plasmid (Brasier et al. Biotechniques, 7:1116-1122 (1989)), under the control of Rous sarcoma virus long terminal repeat (RSV-LTR) promoter. The pGSVLacZ vector contains the simian virus (SV40) large tumor nuclear location signal fused to the lacZ gene (nls .beta.-gal) (Bonnerot et al., Proc. Natl. Acad. Sci. U.S.A., 84:6795-6799 (1987)) (gift from Dr. Claire Bonnerot, Institut Pasteur, Paris, France), inserted into a pGEM1-plasmid. Nuclear staining identifies the exogenous construct designed to permit nuclear translocation, and thus distinguishes expression of the transgene from endogenous (cytoplasmic) .beta.-gal activity. Previous concerns (Lim et al., Circulation, 83:2007-2011 (1991)) regarding nonspecificity of blue staining resulting from .beta.-gal are thus eliminated.

Analysis of Luciferase Activity

The magnitude of gene expression was determined by measuring luciferase activity as described previously (Leclerc et al., J. Clin. Invest., 90:936-944 (1992)) using the Luciferase Assay System (Promega, Madison, Wis.). Briefly, frozen arteries were homogenized and dissolved in 300 .mu.l of Cell Culture Lysis Reagent (Promega) containing 1 mg/ml bovine serum albumin. Three different 20-.mu.l aliquots prepared from each transfected specimen were mixed in a sample tube with 100 .mu.l of

Luciferase Assay Reagent (Promega, Madison, Wis.) and inserted into a luminometer (Model 20e, Turner Design, Sunnyvale, Calif.) that reports results on a scale established to yield as low as $10.\text{sup.}-3$ Turner light units (TLU). The specimen's total luciferase activity was calculated from the mean of the three aliquots analyzed. The luciferase values were in the linear range of a standard curve derived from samples with a known amount of luciferase (Sigma, St. Louis, Mo., catalogue #L9009). The lyophilized luciferase was, according to the manufacturer's instructions, dissolved in sterile water and further diluted in Cell Culture Lysis Reagent with 1 mg/ml bovine serum albumin. The following equation was used to convert TLU into pg luciferase: Luciferase [pg]=-0.08+0.051 TLU. Using this formula, 100 TLU corresponds to 5.0 pg of luciferase. It must be noted that the specific activity of luciferase standards from different vendors can vary considerably (Wolff, et al., Biotechniques, 11:474-485 (1991)); therefore, direct comparisons of luciferase reported by different groups must be made with caution, especially when the origin of the standard used is not specified.

Percutaneous Transfection

Percutaneous gene transfer experiments with the luciferase gene were performed in 13 rabbits using a catheter with a balloon to which a 20 .mu.m hydrogel coating had been applied and which was advanced through a 5 F teflon sheath. The balloon was advanced beyond the distal tip of the sheath, coated with 130 .mu.g luciferase DNA, and pulled back into the sheath to protect the balloon from subsequent contact with blood. The sheath and the angioplasty catheter were then introduced via the right carotid artery and advanced to the left common lilac artery under fluoroscopic control. The balloon catheter was advanced 2 cm further (beyond the distal sheath tip) into the external lilac artery and inflated there for 1 or 5 min. Following balloon deflation, the catheter system was removed. In 10 animals, the transfected external lilac artery as well as the contralateral control artery were removed 3 days later, weighed, and assayed for luciferase activity. In 3 additional animals, which had been transfected for 5 min. only, the arteries were excised 14 days after gene transfer. In these 3 animals we also removed the left femoral artery to check for luciferase expression directly downstream of the transfected segment.

Results

Luciferase expression was detected in all 10 (100%) percutaneously transfected arteries excised after 3 days, whether inflated for 5 min (386.+-.299 TLU, n=5) or 1 min (113.+-.59 TLU, n=5).

Three additional animals, in which balloons were inflated for 5 min only, were sacrificed after 14 days. Individual luciferase expression was 152.6, and 16 TLU, respectively (mean=58.+-.47 TLU). In this series, we also measured luciferase in the adjacent femoral artery, which was not inflated. Luciferase expression in all these arteries was undistinguishable from background activity (mean 0.04.+-.0.29 TLU).

The findings demonstrate that endoluminal vascular gene transfer can be achieved successfully and consistently with pure DNA applied to a standard angioplasty catheter balloon coated with hydrogel polymer. The hydrogel provides the absorbable medium to which one may apply a solution of pure DNA. Drying of the gel results in a layer of concentrated DNA which is then transferred to the arterial wall as the balloon contacts the arterial wall coincident with balloon inflation. Experiments with radiolabeled DNA established that 97% of DNA applied in aqueous solution to the hydrogel-coated balloon was still present on the balloon after drying of the gel. Autoradiograms of the arterial wall demonstrated that inflation of the hydrogel balloon results in DNA uptake which is distributed across the full thickness of the arterial wall. DNA was shown to penetrate the intact internal elastic lamina and was distributed intracellularly as well as extracellularly.

Despite elimination of accessory transfection vehicles in this example, both the frequency of successful transfection and the magnitude of reporter gene expression achieved were superior to that previously reported from our laboratory (Leclerc, et al., J. Clin Invest., 90:936-944 (1992)) and comparable to the results achieved by others (Chapman, et al., Circ. Res., 71:27-33 (1992) and Lim, et al., Circulation, 83:2007-2011 (1991)) using alternative delivery schemes. The success rate of transfection in our rabbit model as measured by expression of the luciferase transgene was 100% (37 of 37 artery segments), even in those cases in which the inflation time was reduced to one minute. The duration of inflation within a range from 10 to 30 minutes did not have significant impact on transfection efficiency, a feature which would be expected to facilitate human arterial, particularly coronary, gene transfer.

EXAMPLE 2

Induction of Angiogeneses In Vivo

Methods

Animal Model (FIG. 1).

The angiogenic response to transfection of the gene for vascular endothelial growth factor (VEGF) was investigated using a rabbit ischemic hindlimb model. Takeshita, et al., J. Clin. Invest., 93:662-670 (1994) and Pu, et al., J. Invest Surg., (In Press). All protocols were approved by St. Elizabeth's Institutional Animal Care and Use Committee. Male New Zealand White rabbits weighing 4-4.5 kg (Pine Acre Rabbitry, Norton, Mass.) were anesthetized with a mixture of ketamine (50 mg/kg) and acepromazine (0.8 mg/kg) following premedication with xyazine (2.5 mg/kg). A longitudinal incision was then performed, extending inferiorly from the inguinal ligament to a point just proximal to the patella. The limb in which the incision was performed--right versus left--was determined at random at the time of surgery by the surgeon. Through this incision, using surgical loops, the femoral artery was dissected free along its entire length; all branches of the femoral artery, including the inferior epigastric, deep femoral, lateral circumflex and superficial epigastric arteries, were also dissected free. After further dissecting the popliteal and saphenous arteries distally, the external lilac artery as well as all of the above arteries were ligated. Finally, the femoral artery was completely excised from its proximal origin as a branch of the external lilac artery, to the point distally where it bifurcates into the saphenous and popliteal arteries. Once the femoral artery is excised, thrombotic occlusion of the external lilac artery extends retrograde to its origin from the common lilac (FIG. 1(a), arrow). As a result, the blood supply to the distal limb is dependent on the collateral arteries which may originate from the internal lilac artery. Accordingly, direct arterial gene transfer of VEGF was performed in to the internal lilac artery of the ischemic limb. Post-operatively, all animals were closely monitored. Analgesia (levorphanol tartrate 60 mg/kg, Roche Laboratories, Nutley, N.J.) was administered subcutaneously as required for evidence of discomfort throughout the duration of the experiment. Prophylactic antibiotics (enrofloxacin 2.5 mg/kg, Miles, Shawnee Mission, Kans.) was also administered subcutaneously for a total of 5 days post-operatively.

Plasmids and Smooth Muscle Cell (SMC) Transfection in Vitro.

Complementary DNA clones for recombinant human VEGF.sub.165, isolated from cDNA libraries prepared from HL60 leukemia cells, were assembled into a mammalian expression vector containing the cytomegalovirus promoter. Leung, et al., Science, 246:1306-1309 (1989). The biological activity of VEGF.sub.165 secreted from cells transfected with this construct (phVEGF.sub.165) was

previously confirmed by the evidence that media conditioned by transfected human 293 cells promoted the proliferation of capillary cells. Leung, et al., Science, 246:1306-1309 (1989).

To evaluate expression of phVEGF.sub.165 in vascular cells, rabbit arterial smooth muscle cells (SMCs) were transfected in vitro. Cells were cultured by explant outgrowth from the thoracic aorta of New Zealand White rabbits. The identity of vascular SMCs was confirmed morphologically using phase contrast microscopy and by positive immunostaining using a monoclonal antibody to smooth muscle .alpha.-actin (Clone 1A4, Sigma, St. Louis, Mo.). Cells were grown in the media (M199, GIBCO BRL, Gaithersberg, Md.) supplemented with 10% fetal bovine serum (FBD, GIBCO BRL). In vitro transfection was performed by incubating SMCs (1.48.times.10.sup.6 cells/10 cm plate) with 11.5 .mu.g of the plasmid DNA and 70 .mu.g of liposomes (Transfection-reagent, Boehringer Mannheim, Indianapolis, Ind.) as previously described. Pickering, et al., Circulation, 89:13-21 (1994). After completion of transfection, media was changed to 10% FBS. Culture supernatant was sampled at 3 days pot-transfection, and was analyzed by ELISA assay for VEGF protein. Houck, et al., J. Biol. Chem. 267:26031-26037 (1992).

The plasmid pGSVLacZ (courtesy of Dr. Claire Bonnerot) containing a nuclear targeted .beta.-galactosidase sequence coupled to the simian virus 40 early promoter (Bonnerot, et al., Proc. Natl. Acad. Sci. USA, 84:6795-6799 (1987)) was used for all the control transfection experiments.

Percutaneous Arterial Gene Transfer in Vitro.

An interval of 10 days between the time of surgery and gene transfer was allowed for post-operative recovery of rabbits and development of endogenous collateral vessels. Beyond this time-point, studies performed up to 90 days post-operatively (Pu, et al., J. Invest. Surg., (In Press)) have demonstrated no significant collateral vessel augmentation. At 10 days post-operatively (day 0), after performing a baseline angiogram (see below), the internal lilac artery of the ischemic limb of 8 animals was transferred with phVEGF.sub.165 percutaneously using a 2.0 mm hydrogel-coated balloon catheter (Slider.TM. with HYDROPLUS,.RTM. Boston Scientific, Watertown, Mass.). The angioplasty balloon was prepared (ex vivo) by first advancing the deflated balloon through a 5 Fr. teflon sheath (Boston Scientific), applying 400 .mu.g of phVEGF.sub.165 to the 20 .mu.m-thick later of hydrogel on the external surface of the inflated balloon, and then retracting the inflated balloon back into the protective sheath. The Sheath and angioplasty catheter were then introduced via the right carotid artery, and advanced to the lower abdominal aorta using a 0.014 inch guidewire (Hi-Torque Floppy II, Advanced Cardiovascular Systems, Temecula, Calif.) under fluoroscopic guidance. The balloon catheter was then advanced out of the sheath into the internal lilac artery of the ischemic limb, inflated for 1 min at 6 atmospheres, deflated, and withdrawn (FIG. 1(a), open arrow). An identical protocol was employed to transfect the internal iliac artery of 9 control animals with the plasmid pGSVLacZ containing a nuclear targeted .beta.-galactosidase sequence. Heparin was not administered at the time of transfection or angiography.

Evaluation of Angiogenesis in the Ischemic Limb.

Development of collateral vessels in the ischemic limb was serially evaluated by calf blood pressure measurement and internal lilac arteriography immediately prior to transfection (day 0), and then in serial fashion at days 10 and 30 post-transfection. On each occasion, it was necessary to lightly anesthetize the animal with a mixture of Ketamine (10 mg/kg) and acepromazine (0.16 mg/kg) following premedication with xyazine (2.5 mg/kg). Following the final 30-day follow-up, the animal was sacrificed, and tissue sections were prepared from the hindlimb muscles in order to perform analysis of capillary density. These analyses are discussed in detail below.

Calf Blood Pressure Ratio.

Calf blood pressure was measured in both hindlimbs using a Doppler Flowmeter (Model 1050, Parks Medical Electronics, Aloha, Oreg.), immediately prior to transfection (day 0), as well as on days 10 and 30. On each occasion, the hindlimbs were shaved and cleaned; the pulse of the posterior tibial artery was identified using a Doppler probe; and the systolic pressure of both limbs was determined using standard techniques. Takeshita, et al., J. Clin. Invest., 93:662-670 (1994). The calf blood pressure ratio was defined for each rabbit as the ratio of systolic pressure of the ischemic limb to systolic pressure of the normal limb.

Selective Internal Iliac Arteriography.

Collateral artery development in this ischemic hindlimb model originates from the internal lilac artery. Accordingly, selective internal lilac arteriography was performed on day 0 (immediately prior to transfection), and again on days 10 and 30 post-transfection as previously described. Takeshita, et al., J. Clin. Invest., 93:662-670 (1994). A 3 Fr. end-hole infusion catheter (Tracker-18, Target Therapeutics, San Jose, Calif.) was introduced into the right common carotid artery through a small cutdown, and advanced to the internal lilac artery at the level of the interspace between the seventh lumbar and the first sacral vertebrae. Following intra-arterial injection of nitroglycerin (0.25 mg, SoloPak Laboratories, Franklin Park, Ill.), a total of 5 ml of contrast media (Isovue-370, Squibb Diagnostics, New Brunswick, N.J.) was then injected using an automated angiographic injector (Medrad, Pittsburgh, Pa.) programmed to reproducibly deliver a flow rate of 1 ml per sec. Serial images of the ischemic hindlimb were then recorded on 105-mm spot film at a rate of 1 film per sec for at least 10 sec. Following completion of arteriography, the catheter was removed and the wound was closed. All of the above-described procedures were completed without the use of heparin.

Morphometric angiographic analysis of collateral vessel development was performed as previously described. Takeshita, et al., J. Clin. Invest., 93:662-670 (1994). A composite of 5-mm.sup.2 grids was placed over the medical thigh area of the 4-sec angiogram. The total number of grid intersections in the medical thigh area, as well as the total number of intersections crossed by a contrast-opacified artery were counted individually by a single observer blinded to the treatment regimen. An angiographic score was calculated for each film as the ratio of grid intersections in the medial thigh.

Capillary Density and Capillary/Myocyte Ratio.

The effect of VEGF gene transfer upon anatomic evidence of collateral artery formation was further examined by measuring the number of capillaries in light microscopic sections taken from the ischemic hindlimbs. Takeshita, et al., J. Clin. Invest., 93:662-670 (1994). Tissue specimens were obtained as transverse sections from the ischemic limb muscles at the time of sacrifice (day 30 post-transfection). Muscle samples were embedded in O.C.T. compound, (Miles, Elkhart, Ind.) and snap-frozen in liquid nitrogen. Multiple frozen sections (5 .mu.m in thickness) were then cut from each specimen on a cryostat (Miles), so that the muscle fibers were oriented in a transverse fashion, and two sections then placed on glass slides. Tissue sections were stained for alkaline phosphate using an indoxyl-tetrazolium method to detect capillary endothelial cells (Ziada, et al., Cardiovasc. Res., 18:724-732 (1984)), and were then counterstained with eosin. Capillaries were counted under a 20x objective to determine the capillary density (mean number of capillaries per mm.sup.2). A total of 20 different fields was randomly selected, and the number of capillaries counted. To ensure that analysis of capillary density was not overestimated due to muscle atrophy, or underestimated due to interstitial edema, capillaries identified at necropsy were also evaluated as a function of myocytes in the

histologic section. The counting scheme used to compute the capillary/myocyte ratio was otherwise identical to that used to compute capillary density.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR), Southern Blot Analysis, and Sequencing of RT-PCR Product.

The presence of human VEGF mRNA was detecting using RT-PCR. Arterial samples were obtained at 5 days post-transfection, and total cellular RNA was isolated using TRI REAGENT (Molecular Research Center, Cincinnati, Ohio) according to the manufacturer's instructions. Extracted RNA was treated with DNase I (0.5 .mu.l, 10 U/.mu.l, RNase-free, Message Clean kit, GenHunter, Boston, Mass.) at 37.degree. C. for 30 min to eliminate DNA contamination. The yield of extracted RNA was determined spectrophotometrically by ultraviolet absorbance at 260 nm. To check that the RNA was not degraded and electrophoresed through a 1% non-denaturing miniagarose gel. 0.5 .mu.g of each RNA sample was used to make cDNA in a reaction volume of 20 .mu.l containing 0.5 mM of each deoxynucleotide triphosphate (Pharmacia, Piscataway, N.J.), 10 mM dithiothreitol, 10 units of RNasin (Promega, Madison, Wis.), 50 mM Tris-HCI (pH 8.3), 75 mM KCL, 3 mM MgCl.sub.2, 1 .mu.g random hexanucleotide primers (Promega), and 200 units of M-MLV reverse transcriptase (GIBCO BRL). For greater accuracy and reproducibility, master mixes for a number of reactions were made up and aliquoted to tubes containing RNA. Reactions were incubated at 42.degree. C. for 1 hr, then at 95.degree. C. for 5 min to terminate the reaction. Twenty .mu.l of diethyl pyrocarbonate (DEPC) water was then added and 5 .mu.l of the diluted reaction (1/8th) was used on the PCR analysis. The optimized reaction in a total volume of 20 .mu.l contained 0.2 mM of each deoxynucleotide triphosphate, 3 mM MgCl.sub.2, 2 .mu.l PCR II buffer (Perkin-Elmer, Norwalk, Conn.; final concentrations, 50 mM KCL, 10 mM Tris-HCL), 5 ng/.mu.l (13.77 pmoles) of each primer, and 0.5 units of AmpliTaq DNA polymerase (Perkin-Elmer). The PCR was performed on a 9600 PCR system (Perkin-Elmer) using microamp 0.2. ml thin-walled tubes. Amplification was for 40-45 cycles of 94.degree. C. for 20 sec, 55.degree. C. for 20 sec, and 72.degree. C. for 20 sec, ending with 5 min at 72.degree. C. To test for false positives, controls were included with no RNA and no reverse transcriptase. A pair of oligonucleotide primers (22 mers) was designed to amplify a 258 bp sequence from the mRNA of human VEGF. To ensure specificity and avoid amplification of endogenous rabbit VEGF, each primer was selected from a region which is not conserved among different species. Sequences of primers used were: 5'-GAGGGCAGAATCATCACGAAGT-3' (sense) SEQ. ID NO:1 ; 5'-TCCTATGTGCTGGCCTTGGTGA-3' (antisense) SEQ. ID NO:2. RT-PCR products were transferred from agarose gels to nylon membranes (Hybond, Amersham, Arlington Heights, Ill.). The probe was 5' end-labelled with T4 polynucleotide kinase and [.beta.-.sup.32 P]ATP (Sambrook, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, (1989)) and hybridized to the nylon filters using Rapid Hybridization buffer (Amersham) according to manufacturer's instructions. To visualize hybridized bands, filters were exposed to X-ray film (Kodak Xar-5).

To confirm the identity of VEGF PCR products. DNA bands were excised from agarose gels, purified using Geneclean (BIO 101, La Jolla, Calif.), and sequenced directly (i.e. without subcloning) using dsDNA Cycle Sequencing System (GIBCO BRL) following the directions of manufacturer. The two VEGF primers used for PCR were 5' end-labeled with [.beta.-.sup.32 P]ATP and T.sub.4 polynucleotide kinase and used as sequencing primers to determine the sequence of both strands of the PCR product.

.beta.-Galactosidase Staining of Transfected lilac Arteries.

To evaluate the efficiency of in vivo arterial gene transfer, .beta.-galactosidase activity was determined

by incubation of arterial segments with 5-bromo-4-chloro-3-indolyl .beta.-D-galactosidase chromogen (X-Gall), Sigma) as previously described. Riessen, et al., Hum. Gene Ther., 4:749-758 (1993). Following staining with X-Gal solution, tissues were paraffin-embedded, sectioned, and counterstained with nuclear fast red. Nuclear localized .beta.-galactosidase expression of the plasmid pGSVLacZ cannot result from endogenous .beta.-galactosidase activity; accordingly, histochemical identification of .beta.-galactosidase within the cell nucleus was interpreted as evidence for successful gene transfer and gene expression. Cytoplasmic or other staining was considered non-specific for the purpose of the present study.

Statistics.

Results were expressed as means.+-.standard deviation (SD). Statistical significance was evaluated using unpaired Student's t test for more than two means. A value of $p < 0.05$ was interpreted to denote statistical significance.

Results

ELISA Assay for VEGF. To test the expression of the plasmid phVEGF.sub.165 in vascular cells, culture supernatant of VEGF-transfected SMCs (1.48.times.10.sup.6 cells/10 cm plate) was sampled at 3 days post-transfection, and analyzed by ELSA for VEGF protein. The media of VEGF-transfected SMCs contained an average of 1.5 .mu.g of VEGF protein (n=3). In contrast, culture media of .beta.-galactosidase-transfected SMCs (n=3) or non-transfected SMCs (n=3) did not contain detectable levels of VEGF protein.

RT-PCR, Southern Blot Analysis, and Sequencing of RT-PCR Product.

To confirm expression of human VEGF gene in transfected rabbit lilac arteries in vivo, we analyzed transfected arteries for the presence of human VEGF mRNA by RT-PCR. As indicated above, to ensure the specificity of RT-PCR for human VEGF mRNA resulting from successful transfection (versus endogenous rabbit VEGF mRNA), primers employed were selected from a region which is not conserved among different species. Arteries were harvested at 5 days post-transfection. The presence of human VEGF mRNA was readily detected in rabbit SMC culture (n=3) and rabbit lilac arteries (n=3) transfected with phVEGF.sub.165. Rabbit lilac arteries transfected with pGSVLacZ (n=3) were negative for human VEGF mRNA (FIG. 2(a)). Southern blot analysis was used to further confirm that the 158 bp bands obtained by RT-PCR did in fact correspond to the region between the two primers (FIG. 2(b)). Direct sequencing of the RT-PCR product document that this band represented the human VEGF sequence (FIG. 2(c)).

Angiographic Assessment.

The development of collateral vessels in the 5 rabbits transfected with phVEGF.sub.165 and 6 rabbits transfected with pGSVLacZ was evaluated by selective internal lilac angiography. FIG. 3 illustrates representative internal lilac angiogram recorded from both control and VEGF-transfected animals. In control animals, collateral artery development in the medial thigh typically appeared unchanged or progressed only slightly in serial angiogram recorded at days 0, 10, and 30 (FIGS. 3(a-c)). In contrast, in the VEGF-transfected group, marked progression of collateral artery was observed between days 10 and 30 (FIGS. 3, (d-f)). Morphometric analysis of collateral vessel development in the media thigh was performed by calculating the angiographic score as described above. At baseline (day 0), there was no significant difference in angiographic score between the VEGF-transfected and control groups (day 0:0.17.+-.0.02 vs 0.20.+-.0.06, p=ns). By day 30, however, the angiographic score in VEGF-

transfected group was significantly higher that in control group (0.47.+-.0.09 vs 0.34.+-.0.10, p<0.05) (FIG. 4(a)).

Calf Blood Pressure Ratio (FIG. 4(b)).

Reduction of the hemodynamic deficit in the ischemic limb following VEGF-transfection was confirmed by measurement of calf blood pressure ratio (ischemic/normal limb). The calf blood pressure ratio was virtually identical in both groups prior to transfection (0.23.+-.0.12 in VEGF-transfected animals, p=ns). By day 10 post-transfection, the blood pressure ration for VEGF-transfected rabbits was significantly higher than for the control rabbits (0.60.+-.0.12 vs 0.32.+-.0.14, p<0.01). At day 30, the blood pressure ratio for the VEGF-transfected group continued to exceed that of controls (0.70.+-.0.08 vs 0.50.+-.0.18, p<0.05).

Capillary Density and Capillary/Myocyte Ratio (FIGS. 4(c), 5).

A favorable effect of VEGF-transfection upon revascularization was also apparent at the capillary level. The medial thigh muscles of the ischemic limbs were histologically examined at day 30 post-transfection. Analysis of capillary density disclosed a value of 233.0.+-.60.9/mm.sup.2 in VEGF-transfected group versus 168.7.+-.31.5/mm.sup.2 in the control group (p<0.05). Analysis of capillary/myocyte ratio disclosed a value of 0.67.+-.0.15 in the VEGF-transfected group versus 0.48.+-.0.10 in the control group (p<0.05).

.beta.-Galactosidase Staining of Transfected Iliac Arteries.

To evaluate the efficiency of in vivo arterial gene transfer, transfected lilac arteries were harvested at 5 days post-transfection, and were used for .beta.-galactosidase histochemical analysis. In arteries transfected with nuclear targeted .beta.-galactosidase, evidence of successful transfection, indicated by dark blue nuclear staining, was observed in only<0.5% of total arterial cells. Arteries transfected with phVEGF.sub.165 were negative for nuclear staining.

EXAMPLE 3

Comparison of Double-Balloon Catheter Technique and Hydrogel-Coated Balloon Catheter Technique

Methods

Recombinant Adenoviral Vectors

Replication-defective recombinant adenoviral vectors, based on human adenovirus 5 serotype, were produced as previously described. Quantin, et al., Proc. Nat. Acad. Sci. USA, 89:2581-2584 (1992); Stratford-Perricaudet, et al., J. Clin. Invest., 90:626-630 (1992); and Rosenfeld, et al., Cell, 68:143-155 (1992). Ad-RSV.beta.gal contains the Escherichia coil lac Z gene and the SV40 early region nuclear localization sequence (nls). The nlslacZ gene encodes a nuclear-targeted .beta.-galactosidase under the control of the Rous sarcoma virus promoter. Ad-RSVmDys, used as a negative control, contains a human "minidystrophin" cDNA under the control of the same promoter. Ragot, et al., Nature, 361:647-650 (1993).

In Vivo Percutaneous Gene Transfer Procedures

All animal procedures were approved by the Institutional Animal Care and Use Committees of Faculte Bichat and St. Elizabeth's Hospital. Gene transfer was performed in the external lilac artery of 29 New Zealand white rabbits under general anesthesia and sterile conditions. Anesthesia was induced with intramuscular acepromazine and maintained with intravenous pentobarbital. Adenoviral stocks were used within 30 minutes of thawing.

1. Double-balloon catheter technique.

In 15 animals, Ad-RSV.beta.gal (2.10.sup.9 to 2.10.sup.10 plaque forming unites {pfu} in 2 ml PBS) was transferred to the right lilac artery, either normal (n=9) or previously denuded (n=6), using a 4 French double-balloon catheter (Mansfield Medical, Boston Scientific Corp., Watertown, Mass.) as previously described. Nabel, et al., Science, 244:1342-1344 (1989). The catheter was positioned in a segment of the artery which lacked angiographically visible side branches. The viral solution was maintained in contact with the arterial wall for 30 min. The left lilac artery of the same 15 animals was used as a control: in 7 animals no catheter was inserted, in 6 animals the endothelium was removed using balloon abrasion, and, in the 2 other animals, a double-balloon catheter was used in infuse Ad-RSVmDys (2.10.sup.9 pfu in 2 ml PBS).

2. Hydrogel-Coated Balloon Catheter Technique.

In 14 animals, a hydrogel-coated balloon catheter was used (Slider.TM. with Hydroplus.RTM., Mansfield Medical, Boston Scientific Corp., Watertown, Mass.). The balloon diameter (either 2.5 or 3.0 mm), was chosen to approximate a 1.0 balloon/artery ratio based on caliper measurement of magnified angiographic frames. Ad-RSV.beta.gal (1-2.10.sup.10 pfu in 100 .mu.1 PBS) was applied to the polymer-coated balloon using a pipette as described above. The catheter was introduced into the right femoral artery through a protective sheath, the balloon was inflated at 1 atm, and the assembly was then advanced over a 0.014" guide wire to the external lilac artery where, after balloon deflation, the catheter alone was advanced 2 cm further and the balloon inflated for 30 minutes at 6 atm (ensuring nominal size of the inflated balloon). The contralateral lilac artery was in each case used as a control: in 9 animals no catheter or virus was introduced, in 2 the endothelium was removed, while in 3 a hydrogel-coated balloon catheter was used to transfer Ad-RSVmDys.

Detection of lacZ Expression in the Arterial Wall.

Three to seven days after transfection, the animals were sacrificed by pentobarbital overdose. To assess nlslacZ gene expression, the arteries were harvested and stained with X-Gal reagent (Sigma) for 6 hours, at 32.degree. C., as previously described. Sanes, et al., EMBO J., 5:3133-3142 (1986). Samples were then either mounted in OCT compound (Miles Laboratories Inc., Ill.) for cryosectioning or embedded in paraffin, cut into 6-.mu.m sections, and counterstained with hematoxylin and eosin or elastic trichrome. Expression of nlslacZ gene was considered positive only when dark blue staining of the nucleus was observed. To determine which cell types within the arterial wall expressed the transgene, immunohistochemical staining of X-Gal-stained arterial sections was performed, using a mouse monoclonal anti-.alpha.-actin primary antibody specific for vascular smooth muscle (HHF-35, Enzo Diagnostics, Farmingdale, N.Y.), and then a polyclonal peroxidase-labeled anti-mouse immunoglobulin G secondary antibody (Signet Laboratories, Dedham, Mass.).

Morphometric Analysis of nlslacZ Gene Expression in the Media.

For each transfected iliac artery, at least 2 samples were taken from the target-zone, and from each sample, at least 3 sections were examined by light microscopy after X-gal staining. Due to the

heterogeneity of .beta.-galactosidase activity on gross examination, the percentage of transfected medial cells per artery section was determined in regions showing high .beta.-galactosidase activity by counting stained versus total nuclei. The total numbers of studied medial cells were 14.10.sup.3 (n=50 sections) in the double-balloon catheter and the hydrogel-coated balloon catheter groups respectively.

Detection of Remote.sup.nlslacZ Gene Transfer and Expression.

Tissue samples from liver, brain, testes, heart, lungs, kidneys, contralateral limb skeletal muscle, and arterial segments adjacent to the treated arterial site were harvested immediately after sacrifice. For each specimen, nlslacZ gene presence and expression were assessed by polymerase chain reaction (PCR) and histochemistry (X-gal staining) respectively.

For PCR, genomic DNA was extracted from tissues by standard techniques. DNA amplification was carried out using oligodeoxynucleotide primers designed to selectively amplify Ad-RSV.beta.gal DNA over endogenous .beta.-galactosidase gene by placing one primer in the adenovirus sequence coding for protein 9 and the other primer in the lacZ sequence (5'-AGCCCGTCAGTATCGGCGGAATTC-3' (SEQ ID NO:3) and 5'-CAGCTCCTCGGTCACATCCAG-3' (SEQ ID NO:4) respectively, Genset, Paris, France). The reactions were performed in a DNA thermocycler (GeneAmp PCR System 9600, Perkin Elmer Cetus, Norwalk, Conn.) following 2 different protocols: a hold at 95.degree. C. for 3 min, 35 or 45 cycles of 95.degree. C. for 30 s, 65.degree. C. for 40 s, and 72.degree. C. for 1 min, then a final extension at 72.degree. C. for 5 min. When PCR was performed on plasmid DNA containing the nlslacZ gene used for the preparation of the adenoviral vector, or on positive liver samples obtained by deliberate systemic injection of Ad-RSV.beta.gal, the amplification reaction produced a 700 bp DNA fragment. To determine sensitivity of these procedures, DNA was extracted from liver of uninfected rabbits, aliquoted into several tubes, and spiked with dilutions of the plasmid containing the nlslacZ gene and used as a positive control. Following the amplification protocols described above, it was determined that the 35- or 45-cycle PCR could detect one copy of the nlslacZ gene in 3.102 and 3.104 cells respectively. DNA extractions and amplifications were performed simultaneously and in duplicate for studied tissues and positive controls.

Each tissue sample was also processed for histochemical analysis following the same protocol described for the arteries. For each specimen, at least 3 different segments were obtained, embedded in paraffin, and cut into at least 5 sections. Sections were counterstained with hematoxylin and eosin, and examined by light microscopy for the presence of deep blue nuclei indicative of .beta.-galactosidase expression. The number of positive cells as well as the total number of cells were counted. The total number of cells examined per sample ranged from 25.10.sup.3 to 115.10.sup.3.

Statistics

Results are expressed as mean.+-.standard deviation (SD). Comparisons between groups were performed using Student's t test for unpaired observations. A value of $p<0.05$ was accepted to denote statistical significance.

Results

Histological and Histochemical Analyses of Transfected Arteries Following Double-Balloon Catheter Delivery

Gross examination of all the arteries (n=15) following X-gal staining showed punctiform, heterogeneous, blue staining on the luminal aspect of the arteries, always limited to the area between

the two balloons. For the 9 normal arteries, microscopic examination disclosed dark blue nuclear staining, confined entirely to the endothelium. In contrast, when endothelial abrasion preceded transfection (n=6), X-gal staining imparted a mottled appearance to the luminal aspect of the artery. In these cases, microscopic examination showed that the endothelium had been removed and that the site of X-gal staining was subjacent to the intact internal elastic lamina, involving sparse medial cells. The identity of the transfected medial cells as smooth muscle cells was confirmed by immunohistochemical staining with monoclonal anti-.alpha.-actin antibody. Control arteries showed no nuclear blue staining.

Histological and Histochemical Analysis of Transfected Arteries Following Hydrogel-Coated Balloon Catheter Delivery

Gross examination of all the arteries after X-gal staining (n=14) showed dark blue, heterogeneous staining of the transfected site with a sharp boundary between the transfected segment and the bordering proximal and distal segments. Microscopic examination showed no residual intact endothelium; the continuity of the internal elastic lamina, in contrast, appeared preserved without apparent disruption. In the areas showing evidence of .beta.-galactosidase activity on gross examination, light microscopic examination revealed nearly continuous layers of cells with dark blue nuclear staining, generally limited to the superficial layers of the media; occasionally, sparsely distributed cells from deeper layers of the media expressed the transgene as well. Staining with monoclonal anti-.alpha.-actin antibody confirmed that transfected cells were vascular smooth muscle cells. No evidence of nuclear .beta.-galactosidase activity was seen in control arteries.

Morphometric Analysis of nlslacZ Gene Expression in the Media.

The percentage of transduced cells per artery section in regions showing high .beta.-galactosidase activity was significantly higher in the hydrogel-coated balloon catheter group than in the double-balloon catheter group (6.1.+-.2.3% vs. 0.4.+-.0.6%, p<0.0001).

Detection of Remote lacZ Gene Transfer and Expression in Other Organs

In all animals of both groups, gross and microscopic examination of X-gal stained tissue samples from liver, brain, testes, heart, lungs, kidneys, contralateral limb skeletal muscle, and arterial segments adjacent to the treated arterial site failed to show expression of nuclear-targeted .beta.-galactosidase, except in the liver of one rabbit in the double-balloon catheter group which disclosed a limited area of nuclear and peri-nuclear blue staining. In this area, less than 1/2.10.sup.3 cells expressed .beta.-galactosidase. In a few macrophages limited to samples removed from the lungs and testes of one hydrogel-coated balloon catheter treated rabbit, blue staining of the cytoplasm without nuclear staining was observed; the exclusively cytoplasmic location of .beta.-galactosidase activity in these cases, however, suggested that staining resulted from endogenous .beta.-galactosidase.

All of the above tissue samples were then screened by PCR. When the PCR was run for 35 cycles, the presence of DNA sequence specific for Ad-RSV.beta.gal was non-detectable, including in tissue samples from those animals with the highest percentage of transfected lilac arterial cells. Using an optimized protocol of 45 cycles, however, PCR was positive in the single liver that was observed to express .beta.-galactosidase, but in none of the other tissues.

This invention has been described in detail including the preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements thereon without departing from the spirit and scope of the invention as set forth in the claims.

HARVARD UNIVERSITY Gazette

The following articles appeared in the May 14, 1998, issue. Brief items have been omitted.

College Admission Yield Is Nearly 80%

Women's Studies in Religion Brings New Voices, Perspectives

Bone Drug Lowers Risk of Heart Disease

Virtual Press Room Open for Harvard Conference on Internet & Society

Notes

Labor Economist Myra Strober to Deliver Feminist Economics Lecture at Radcliffe Institute

Police Blotter

A Life of Service

NewsMakers

Peiser Appointed as Professor at Graduate School of Design

Study Finds that Governmental Procedure To Reduce Litigation Actually Leads to More Lawsuits

Knowles Elected Trustee Of Howard Hughes Medical Institute

Faculty To Meet with South Africa's Desmond Tutu, Truth Commission

'Radrugby': Bruised, Battered, Unbowed

Fragments of a Forgotten Past

FAS Administrative, Professional Prizes Honor Staff

New Harvard Features Service Goes Online

EXHIBIT C-6

Seven Students Win Paine Fellowships

Dental Center's Faculty Practice What They Teach

New Arteries Grown In Diseased Hearts

By William J. Cromie

Gazette Staff

Almost anything Hugh Curtis did gave him a pain in the heart. Even when lying in bed, he felt the stabbing chest pains of angina, a hurtful signal that his heart was not getting enough oxygen.

Curtis underwent a quadruple bypass in 1986, then a single bypass late last year. Surgeons removed veins from his legs and grafted them onto his heart to bypass his blocked coronary arteries. But that didn't solve his problem.

He also received a series of angioplasties, wherein tiny balloons were threaded into his heart's arteries, then inflated. This process pushed the blockages aside, opening his arteries. Five pieces of metal mesh were installed to keep them open, but his coronary arteries closed in other places.

"I couldn't walk very far, couldn't even make my bed," says the 55-year-old resident of Danvers, Mass. "Climbing stairs was out, so was any thought of going on vacation."

Late last year, he was asked by researchers at Beth Israel Deaconess Medical Center in Boston if he wanted to volunteer for an experimental procedure at the Harvard-affiliated hospital. The procedure involved doctors injecting proteins called growth factors into his heart to stimulate growth of new blood around those clogged with plaque.

"I didn't hesitate to give them the go-ahead," Curtis recalls.

The cardiologists threaded a thin hollow tube from his groin into his heart. Through the tube they injected what is called basic fibroblast growth factor, or bFGF.

Four months after the treatment, Curtis is back working full time at a desk job in a printing company. "I no longer take 3-to-6 nitroglycerin tablets a day, and I'm painting the hallway in my house," he says cheerily. "I may never go back to playing racquetball, but I'm leading a normal life, and that's all I'm looking for."

"All his symptoms are gone," says Michael Simons, associate professor of medicine at Harvard Medical School. "He is one of 18 patients who participated in a trial of bFGF. All are now largely without

symptoms such as chest pain, shortness of breath, and fatigue."

Bypassing Bypass Surgery

Eighteen other patients who received heart-artery bypasses got bFGF at the same time. Frank Sellke, an associate professor of surgery at Harvard Medical School, implanted capsules that slowly release the drug at sites where blocked vessels were too small or too diffusely diseased to bypass.

"These patients have undergone treadmill stress tests," Simons comments. "They also have been examined with a new type of magnetic resonance imaging (MRI) that measures blood flow and detects new vessel development. It is too early to scream and shout with success, but we are pleased with the results obtained so far."

"I had an MRI a couple of weeks ago, and it showed new arteries growing and bypassing some blockage," says Curtis. "I'm getting 70 percent blood flow to an area of the heart that was down to 30 percent flow. And there's reason to think things will improve more with time."

John Modugno, 48, received bFGF in February, and his MRI tests also show evidence of new arterial growth. "I feel much better," he says, "although I'm still on drugs and get a little angina at the end of the day."

Tests of bFGF and other growth factors now under way at various research centers raise hopes that newly grown blood vessels will replace arteries choked off by atherosclerosis, thus heading off thousands, maybe millions, of heart failures and heart attacks.

If these tests continue to be successful in humans, they could lead to heart drugs that will be cheaper, safer, and a lot easier on patients than bypass surgery and angioplasty. About a million people undergo such procedures in the United States each year, but they don't always work. As in Hugh Curtis's case, some vessels are too small or located where they can't be bypassed with sections of vein. After arteries have been opened by an inflated balloon or other types of angioplasty, about one-third of them close again, some in a matter of months.

"We once thought people in which neither procedure worked accounted for only a small subgroup of patients," Simons says. "But now we're getting phone calls almost every day, so we must conclude that there are more people with this problem than we imagined."

The revolutionary potential of growth factors, of course, goes far beyond such people. Simons sees it as "having the potential to replace or reduce the use of bypass surgery." The American Heart Association estimates that 500,000 bypasses are performed each year at an average cost of $45,000 per treatment.

Severely blocked coronary arteries cause more than 3 million heart failures a year, and 7 million more people suffer the chest pains of angina. "Growth-factor treatments might be expanded to many, if not all, of these patients," Simons declares.

The Side-Effects Question

Researchers at Beth Israel Deaconess Medical Center initiated such treatments in 1996. Today, seven

teams worldwide work on growing new blood vessels with bFGF and another protein known as vascular endothelial growth factor, or VEGF (see April 23 *Gazette,* page 1).

In a trial conducted at several medical centers, VEGF was given to 17 people whose blocked coronary arteries lay out of reach of angioplasty. Fifteen of the 17 patients showed various levels of improvement.

Jeffrey Isner, a cardiologist at St. Elizabeth's Medical Center in Boston, has used VEGF to grow new vessels around blockages in the leg veins of diabetics. He has treated 30 diabetic patients, as well as five other patients with heart disease.

"Preliminary results look good in both types of disease," Isner says. "This is a very encouraging and exciting area of treatment."

The great promise of bypassing blood-vessel blockages won't become a reality, however, if the growth factors cause severe side effects.

Both bFGF and VEGF lower blood pressure. "That fact limits the amount you can give a person," Simons notes. "But that's something we can work around."

More serious is the possibility of damage to sight caused by overgrowth of blood vessels in the eye. "We have been looking carefully for this, but have not seen any as yet with bFGF," Simons comments. Also, no new blood vessels were seen growing in the eyes of patients treated with VEGF, another encouraging sign.

The most worrisome possibility concerns growth of blood vessels that might nourish small, hidden cancer tumors. Judah Folkman, another Harvard researcher, found that such tumors remain benign unless new blood vessels carry nutrients to them. Once connected to a steady blood supply, tumors grow and spread.

Folkman and Michael O'Reilly developed two exciting new cancer drugs, endostatin and angiostatin, which block rather than promote development of blood vessels.

"We hope that tumor growth can be avoided because we give the growth factor for a very short time and in small amounts," Simons notes. "It's not like we're adding a foreign substance to the body; everyone has such small amounts of bFGF circulating naturally in their bloodstream."

The side-effects issue will be addressed in tests involving larger numbers of patients. Plans call for testing both growth factors on 400 to 500 people at a combination of medical centers throughout the country. Simons expects to start expanded trials of bFGF this summer in a collaboration with Emory University in Atlanta.

A question still to be answered is exactly how new blood vessels form. The bare-bones explanation has bFGF binding to the surface of, then stimulating growth of endothelial cells, those that line the inside of capillaries, the smallest vessels. These cells leave the vessels, migrate to the tip of the capillaries, and form a tube that extends their reach.

Simons's team took startling photos of new vessels growing around blocked arteries in animals. They show small extensions sprouting like twigs on a tree limb, moving around the barricade and reconnecting on the other side.

"It's amazing to see," Simons says. "If we can continue to do the same thing in humans, without deleterious side effects, we have a chance to benefit millions of people."

END

College Admission Yield Is Nearly 80%

Highest in 25 years

Nearly 80 percent of students admitted to the Class of 2002 have chosen to enroll, the highest yield since the early 1970s, according to the Undergraduate Admissions Office. This yield is the best in more than 25 years.

Yield, the percentage of admitted candidates who decide to accept an offer of admission, is considered a measure of a school's competitiveness. Harvard's yield is again, by a wide margin, the highest of the nation's selective colleges. When the final figures are available, the yield could go even higher -- it is already well above last year's yield of 76.3 percent.

The 2,073 students admitted to the Class of 2002 were selected from a pool of 16,819 applicants. For the seventh time in eight years, applications for admission to Harvard and Radcliffe have risen. Last year, 16,597 students applied for the 1,650 places in the entering class.

The substantial rise in the yield means that the Class of 2002 is now full, and it will probably be impossible to admit anyone from the waiting list. In more typical years, the College has been able to admit between 50 and 100 from the waiting list.

"We are extremely pleased that the College has again attracted so many extraordinarily talented students this year," said William R. Fitzsimmons '67, Dean of Admissions and Financial Aid. "With many leading American and international universities recently announcing changes in their financial aid programs designed to compete more aggressively for top students, the leadership of Dean of the Faculty of Arts and Sciences Jeremy Knowles and President Neil Rudenstine allowed Harvard to extend its best welcome to prospective members of the Class of 2002."

The Dean and President reemphasized their unwavering commitment to a strong need-based financial aid program and to the policy of admitting the best students without regard to their financial circumstances. With nearly 70 percent of all undergraduates on financial aid, and with scholarship grants of $45 million, Harvard has always been a leader in financial aid.

Dean Knowles stated in February, "We shall set no limit on the financial resources necessary to make Harvard College fully accessible to all students of promise. . . Students who are admitted to next fall's entering class will receive competitively supportive offers, and financial aid will be tailored flexibly and individually."

James S. Miller, director of financial aid, and his staff were available weekdays from 8 a.m. to 8 p.m. and several Saturdays for the month of April, and talked with an unprecedented number of students and parents about their financial aid awards. "Jim and his staff worked extremely hard to make it possible for

EXHIBIT C-7

THE LANCET

Early reports

Clinical evidence of angiogenesis after arterial gene transfer of phVEGF₁₆₅ in patient with ischaemic limb

Jeffrey M Isner, Ann Pieczek, Robert Schainfeld, Richard Blair, Laura Haley, Takayuki Asahara, Kenneth Rosenfield, Syed Razvi, Kenneth Walsh, James F Symes

Summary

Background Preclinical findings suggest that intra-arterial gene transfer of a plasmid which encodes for vascular endothelial growth factor (VEGF) can improve blood supply to the ischaemic limb. We have used the method in a patient.

Methods Our patient was the eighth in a dose-ranging series. She was aged 71 with an ischaemic right leg. We administered 2000 μg human plasmid phVEGF₁₆₅ that was applied to the hydrogel polymer coating of an angioplasty balloon. By inflating the balloon, plasmid DNA was transferred to the distal popliteal artery.

Findings Digital subtraction angiography 4 weeks after gene therapy showed an increase in collateral vessels at the knee, mid-tibial, and ankle levels, which persisted at a 12-week view. Intra-arterial doppler-flow studies showed increased resting and maximum flows (by 82% and 72%, respectively). Three spider angiomas developed on the right foot/ankle about a week after gene transfer; one lesion was excised and revealed proliferative endothelium, the other two regressed. The patient developed oedema in her right leg, which was treated successfully.

Interpretation Administration of endothelial cell mitogens promotes angiogenesis in patients with limb ischaemia.

Lancet 1996; **348**: 370–74

Introduction

Among the growth factors that promote angiogenesis, vascular endothelial growth factor (VEGF),[1] also known as vascular permeability factor,[2] and vasculotropin,[3] is specifically mitogenic for endothelial cells. The first exon of the VEGF gene includes a secretory signal sequence that permits the protein to be secreted naturally from intact cells.[3] We have shown[4,5] that arterial gene transfer of naked DNA encoding for secreted protein yielded physiological levels of protein despite low transfection efficiency. Site-specific gene transfer of plasmid DNA encoding the 165-aminoacid isoform of human VEGF (phVEGF₁₆₅) applied to the hydrogel polymer coating of an angioplasty balloon,[6] and delivered percutaneously to the iliac artery of rabbits in which the femoral artery had been excised to cause unilateral hindlimb ischaemia led to

Departments of Medicine, Biomedical Research, Radiology, and Surgery, St Elizabeth's Medical Center, Tufts University School of Medicine, Boston, Massachusetts, USA (Jeffrey M Isner MD, Ann Pieczek RN, Robert Schainfeld DO, Richard Blair MD, Laura Haley BS, Takayuki Asahara MD, Kenneth Rosenfield MD, Syed Razvi MD, Kenneth Walsh PhD, James F Symes MD)

Correspondence to: Dr Jeffrey M Isner, St Elizabeth's Medical Center, Boston, MA 02135, USA

development of collateral vessels and increased capillary density, improved calf blood-pressure ratio (ischaemic/normal limb) and increased resting and maximum vasodilator-induced blood flow.[4,6] We now use this strategy in the ischaemic limb of a patient.

Patient and methods

Patient

A 70-year-old non-diabetic woman was referred for gangrene of the right great toe. About a year earlier, the patient had cramping right-foot pain; several corns were removed, the was given intramuscular cortisone, prescribed ibuprofen, and fitted with shoe inserts. Symptoms worsened and the patient received oxycodone, hydrocodone, and a fentanyl patch. The great toe lesion progressed to gangrene, and the second and third toes became compromised. She had no palpable pedal pulses of the right limb. Ankle-brachial index of the ischaemic limb was 0·26. Arteriography revealed a 40% stenosis of the proximal popliteal artery, and occlusion of the peroneal, anterior tibial, and posterior tibial arteries midway to the foot. Surgical exploration of the distal right limb failed to identify a suitable site for a bypass.

The patient was suitable for arterial gene therapy according to a protocol[7] approved by the Human Institutional Review Board and Institutional Biosafety Committee of our centre, as well as the Recombinant DNA Advisory Committee of the National Institutes of Health and the US Food and Drug Administration.

Plasmid DNA

phVEGF₁₆₅ consists of a eucaryotic PUC 118 expression vector into which cDNA encoding the 165-aminoacid isoform of VEGF has been inserted.[1] A 763 basepair cytomegalovirus promoter/enhancer is used to drive VEGF expression. The PUC 118 vector includes an SV40 polyadenylation sequence, an *Escherichia coli* origin of replication, and the β-lactamase gene for ampicillin resistance. The plasmid was prepared in the Human Gene Therapy Laboratory at our centre from cultures of phVEGF₁₆₅-transformed *E coli*, purified with a Qiagen-tip 2500 column, precipitated with isopropanol, washed with 70% ethanol, and dried on a Speed Vac. The purified plasmid was reconstituted in sterile saline, stored in vials, and pooled for quality control analyses (absorbance at wavelengths of 260 and 280 nm to document ratio between 1·75 and 1·85; limulus amoebocyte lysate gel-clot assay [BioWhittaker] to establish bacterial endotoxin levels below 5 endotoxin units per kg bodyweight; microbial cultures; southern blot for level of contaminating genomic *E coli* DNA; and ethidium bromide staining after agarose-gel electrophoresis to confirm that over 90% of the nucleic acid was in the closed, circular supercoiled form). To confirm the identity of the prepared plasmid, the VEGF-coding region from each pooled batch was resequenced (Applied Biosystem 373A).

Percutaneous arterial gene transfer

Arterial gene transfer was done with a hydrogel-coated balloon-angioplasty-catheter (Boston Scientific).[6] A sterile pipette was used to apply 2000 μg plasmid DNA at 10·3 μg/μL in 194·2 μL

EXHIBIT D

DISCLOSURES

Growth factors can be utilized to induce the growth of "hard tissue" or bone and "soft tissues" like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basic)(FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 (OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors, and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound, by electricity, by heat, by selected in vivo chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor can also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such a small incision comprises minor surgery which can often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

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In another embodiment of the invention, genetically produced living material is used to form an implant in the bone of a patient. The DNA structure of a patient is analyzed from a sample of blood or other material extracted from a patient and a biocompatible tooth bud 122 (FIG. 3) is produced. The bud 122 is placed in an opening 123 in the alveolar bone and packing material is placed around or on top of the bud 122. The size of opening 123 can vary as desired. The packing around bud 122 can comprise HAC 124, hydroxyapatite, blood, growth factors, or any other desirable packing material. The bud 122 grows into a full grown tooth in the same manner that tooth buds which are in the jaws of children beneath baby teeth grow into full sized teeth. Instead of bud 122, a quantity of genetically produced living material which causes bud 122 to form in the alveolar bone can be placed at a desired position in the alveolar bone such that bud 122 forms and grows into a full sized tooth. Instead of forming an opening 123, a needle or other means can be used to simply inject the genetically produced living material into a selected location in the alveolar bone. As would be appreciated by those skilled in the art, genetically produced materials can be inserted in the body to cause the body to grow, reproduce, and replace leg bone, facial bone, and any other desired soft and hard tissue in the body.

EVIDENCE APPENDIX

ITEM NO. 4

Supplemental Declaration of Dr. Andrew E. Lorincz
filed November 15, 2004

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: James P. Elia)
)   Group Art Unit: 1646
Serial No.: 09/064,000)
)   Examiner: Elizabeth Kemmerer, Ph.D.
Filed: April 21, 1998)
)
For: METHOD AND APPARATUS)
     FOR INSTALLATION OF)
     DENTAL IMPLANT)

LETTER

MAIL STOP AF
Commissioner for Patents
P.O. Box 1450
Arlington, VA 22313-1450

Sir:

Enclosed herewith, please find the Supplemental Declaration of Andrew E. Lorincz, M.D.

This Supplemental Declaration is being submitted in an effort to reduce the number of issues in the instant application and thereby expedite the prosecution thereof.

Respectfully submitted,

Date: <u>November 9, 2004</u>

Gerald K. White

Gerald K. White
Reg. No. 26,611

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Re Application of: James P. Elia)
) Group Art Unit: 1646
Serial No.: 09/064,000)
) Examiner: Elizabeth C. Kemmerer, Ph.D.
Filed: April 21, 1998)
)
For: METHOD FOR GROWTH)
OF SOFT TISSUE)

SUPPLEMENTAL DECLARATION
OF ANDREW E. LORINCZ, M.D.

I Andrew E. Lorincz declare as follows:

1.   I reside at 13820 NW County Rd 235, Apt 8, Alachua, FL 32616-2098.

2.   This Supplemental Declaration is submitted in addition to my previously submitted Declaration in this application, dated February 12, 2001, and makes no changes to such previous Declaration.

3.   My Curriculum Vitae is attached as Exhibit A to my previous Declaration.

4.   In addition to the information set forth in my CV, I provide the following information:

I am familiar with stem cell technology, including bone marrow preparation.

Several publications involving cells are included in my CV; namely, <u>Fluorescent</u> <u>Microscopy of DES-induced Morphologic Transformation in Unfixed, Cultured</u> <u>Cells</u> and <u>Biochemical Genetic Defects.</u>

I performed an unreported study involving assessing stem cell infusion into patients to correct Hurler's Syndrome by transplanting cord blood stem cells. My CV is replete with references to Hurler's Syndrome, as well as other cellular studies.

I am currently Chairman of Vitalflor, a company involved in the observation of cells in the microscopy of vitally stained living organisms, including cells. In this regard, I was granted U.S. Patent Nos. 5,812,314; 6,239,906 B1; and 6,567,214 B2, all of which relate to special stains useful in assessments.

5.   I have read and understood the disclosures of the above-referenced patent application at page 20, line 10 through page 21, line 15; at page 37, lines 19-25; at page 44, line 19 through page 46, line 16; and at page 47, line 22 through page 48, line 15. A copy of such disclosures is attached hereto as Exhibit A.

6.   I note that the disclosures referenced in above Paragraph 5 relate to using a growth factor for promoting the growth of soft tissue, and more specifically, to a method of using a cell, such as a stem cell, to grow soft tissue, such as an artery.

7.   I am aware of and have considered the definition of *growth factor* in the specification of the above-referenced patent application at page 20, line 10 through page 21, line 15. Such definition is set forth in Exhibit B. Also included in Exhibit B is a definition from the medical dictionary, MEDLINE plus: Merriam-Webster Medical Dictionary, a service of the U.S. NATIONAL LIBRARY OF MEDICINE and the NATIONAL INSTITUTES OF HEALTH. I find that the dictionary definition is consistent with that contained at page 20, line 10 through page 21, line 15 of the above-referenced patent application. I believe

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that both definitions are appropriate for use in the field of tissue growth and would be understood by one skilled in the medical arts. Accordingly, I am adopting and utilizing the definition contained in the patent application throughout this declaration.

8. I have read and understood the claims set forth in Exhibit C and have been informed that such claims are present in the above-referenced patent application. It is my opinion that those skilled in the medical arts, reading such claims would understand that cells including stem cells, are species of living organisms.

9. The publication in attached Exhibit D illustrates that placement of a growth factor, including cells, and more specifically, stem cells, in a human patient forms soft tissue, such as an artery. This publication reports work performed by reputable, skilled scientists and reputable organizations in the medical arts. Consequently, I believe that these reports would be recognized as clearly valid by one of ordinary skill in the medical arts because they report the results of scientific tests conducted by competent, disinterested third parties with use of proper scientific controls.

10. Based upon above Paragraphs 5-9, it is my opinion that introducing a growth factor, including cells, and more specifically, stem cells, in the body of a human patient will predictably result in the growth of soft tissue, such as an artery.

11. Based upon above Paragraphs 5-8, it is my opinion that one skilled in the medical arts, armed with the knowledge in such paragraphs, would be able to practice the method set forth in Exhibit C without need for resorting to undue experimentation.

12. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 11-8-04

Andrew E. Lorincz

4

SUPPLEMENTAL
EXHIBIT A

DISCLOSURES

APPLICATION
SERIAL NO. 09/064,000

SUPPLEMENTAL EXHIBIT A

DISCLOSURES
APPLICATION SERIAL NO. 09/064,000

PAGE 20, LINE 10 – PAGE 21, LINE 15

Growth factors can be utilized to induce the growth of "hard tissue" or bone and "soft tissues" like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basis (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

- A1 -

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor an also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which an often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 37, LINES 19-25

Multifactorial and nonspecific cells (such as stem cells and germinal cells) can provide the necessary in vivo and in vitro cascade of genetic material once an implanted master control gene's transcription has been activated. Likewise, any host cell, clone cell, cultured cell, or cell would work. Genetic switches (such as the insect hormone ecdysone) can be used to control genes inserted into humans and animals. These gene switches can also be used in cultured cells or other cells. Gene switches govern whether a gene is on or off making possible precise time of gene activity.

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in connection with any of the implant techniques of the invention, it is understood that a cell nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated (taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which

promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth factors (or other genetic material) are placed. Once the new muscle and blood vessels have

grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

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Organs and/or tissues can be formed utilizing the patient's own cells. For example, a skin cell(s) is removed from the intraoral lining of a cheek. The cell is genetically screened to identify DNA damage or other structural and/or functional problems. Any existing prior art genetic screening technique can be utilized. Such methods can utilize lasers, DNA probes, PCR, or any other suitable device. If the cell is damaged, a healthy undamaged cell is, if possible, identified and selected. If a healthy cell can not [sic] be obtained, the damaged cell can be repaired by excision, alkylation, transition or any other desired method. A growth factor(s) is added to the cell to facilitate dedifferentiation and then redifferentiation and morphogenesis into an organ or function specific tissue. Any machine known in the art can be used to check the genetic fitness of the organ and its stage of morphogenesis. A cell nutrient culture may or may not be utilized depending on the desired functional outcome (i.e., growth of an artery, of pancreatic Islet cells, of a heart, etc.) or other circumstances. Replantation can occur at any appropriate stage of morphogenesis. The foregoing can be repeated without the patient's own

cells if universal donor cells such a [sic] germinal cells are utilized. Germinal cells do not require a dedifferentiation. They simply differentiate into desired tissues or organs when properly stimulated. Similarly, the DNA utilized in the foregoing procedure can come from the patient or from any desired source.

During reimplantation one of the patient's own cells is returned to the patient. During implantation, a cell not originally obtained from the patient is inserted on or in the patient.

In the example above, if germinal cells (and in some case, stem cells) are utilized a direct differentiation and morphogenesis into an organ can occur in vivo, ex vivo, or in vitro.

SUPPLEMENTAL
EXHIBIT B

DEFINITIONS

SUPPLEMENTAL EXHIBIT B

DEFINITIONS

PAGE 20, LINE 10 – PAGE 21, LINE 15

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by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor an also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which an often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

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> **Growth factor:** a substance (as a vitamin B_{12} or an interleukin)
> that promotes growth and especially cellular growth

SUPPLEMENTAL
EXHIBIT C

CLAIMS

EXHIBIT C

CLAIMS
APPLICATION SERIAL NO. 09/064,000

382. A method for producing a desired soft tissue in a body of a human patient comprising:

    (a)   Placing cells in said body of said human patient;

    (b)   Forming a bud in said body of said human patient; and

    (c)   Growing said desired soft tissue from said bud.

383. The method of claim 382, wherein said cells are multifactorial and non-specific.

384. The method of claim 383, wherein said cells comprise stem cells.

385. The method of claim 382 further comprising forming a new artery.

386. The method of claim 383 further comprising forming a new artery.

387. The method of claim 382, wherein said soft tissue comprises mesodermal tissue.

388. The method of claim 382, wherein said soft tissue comprises an artery.

SUPPLEMENTAL
EXHIBIT D

PUBLICATIONS

Repair of Infarcted Myocardium by Autologous Intracoronary Mononuclear Bone Marrow Cell Transplantation in Humans

Bodo E. Strauer, MD; Michael Brehm, MD; Tobias Zeus, MD; Matthias Köstering, MD; Anna Hernandez, PhD; Rüdiger V. Sorg, PhD; Gesine Kögler, PhD; Peter Wernet, MD

Background—Experimental data suggest that bone marrow–derived cells may contribute to the healing of myocardial infarction (MI). For this reason, we analyzed 10 patients who were treated by intracoronary transplantation of autologous, mononuclear bone marrow cells (BMCs) in addition to standard therapy after MI.

Methods and Results—After standard therapy for acute MI, 10 patients were transplanted with autologous mononuclear BMCs via a balloon catheter placed into the infarct-related artery during balloon dilatation (percutaneous transluminal coronary angioplasty). Another 10 patients with acute MI were treated by standard therapy alone. After 3 months of follow-up, the infarct region (determined by left ventriculography) had decreased significantly within the cell therapy group (from 30 ± 13 to $12\pm7\%$, $P=0.005$) and was also significantly smaller compared with the standard therapy group ($P=0.04$). Likewise, infarction wall movement velocity increased significantly only in the cell therapy group (from 2.0 ± 1.1 to 4.0 ± 2.6 cm/s, $P=0.028$). Further cardiac examinations (dobutamine stress echocardiography, radionuclide ventriculography, and catheterization of the right heart) were performed for the cell therapy group and showed significant improvement in stroke volume index, left ventricular end-systolic volume and contractility (ratio of systolic pressure and end-systolic volume), and myocardial perfusion of the infarct region.

Conclusions—These results demonstrate for the first time that selective intracoronary transplantation of autologous, mononuclear BMCs is safe and seems to be effective under clinical conditions. The marked therapeutic effect may be attributed to BMC-associated myocardial regeneration and neovascularization. (*Circulation.* 2002;106:1913-1918.)

Key Words: myocardial infarction ∎ cell transplantation, intracoronary ∎ angiogenesis ∎ bone marrow ∎ myogenesis

Remodeling of the left ventricle after myocardial infarction (MI) represents a major cause of infarct-related heart failure and death. This process depends on acute and chronic transformation of both the necrotic infarct region and the non-necrotic, peri-infarct tissue.[1,2] Despite application of pharmacotherapeutics and mechanical interventions, the cardiomyocytes lost during MI cannot be regenerated. The recent finding that a small population of cardiac muscle cells is able to replicate itself is encouraging but is still consistent with the concept that such regeneration is restricted to viable myocardium.[3]

In animal experiments, attempts to replace the necrotic zone by transplanting other cells (eg, fetal cardiomyocytes or skeletal myoblasts) have invariably succeeded in reconstituting heart muscle structures, ie, myocardium and coronary vessels. However, these cells fail to integrate structurally and do not display characteristic physiological functions.[4-7] Another approach to reverse myocardial remodeling is to repair myocardial tissue by using bone marrow–derived cells. Bone

marrow contains multipotent adult stem cells that show a high capacity for differentiation.[8-10] Experimental studies have shown that bone marrow cells (BMCs) are capable of regenerating infarcted myocardium and inducing myogenesis and angiogenesis; this leads in turn to amelioration of cardiac function in mice and pigs.[11-14] However, procedures based on this phenomenon remain largely uninvestigated in a human clinical setting.

An investigation of one patient receiving autologous skeletal myoblasts into a postinfarction scar during coronary artery bypass grafting revealed improvement of contraction and viability 5 months afterward.[15] Autologous mononuclear BMCs transplanted in a similar surgical setting showed long-term improvement of myocardial perfusion in 3 of 5 patients and no change in 2 patients.[16] However, such studies entail a surgical approach and are therefore associated with well-known perioperative risks. Moreover, this surgical procedure cannot be used with MI. We therefore looked for a nonsurgical, safer mode for transplanting autologous cells

Figure 1. Procedure of cell transplantation into infarcted myocardium in humans. a, The balloon catheter enters the infarct-related artery and is placed above the border zone of the infarction. It is then inflated and the cell suspension is infused at high pressure under stop-flow conditions. b, In this way, cells are transplanted into the infarcted zone via the infarct-related vasculature (red dots). Cells infiltrate the infarcted zone. Blue and white arrows suggest the possible route of migration. c, A supply of blood flow exists within the infarcted zone.[36] The cells are therefore able to reach both the border and the infarcted zone.

into postinfarction tissue. A pilot study from our group demonstrated that intracoronary transplantation of autologous mononuclear BMCs 6 days after MI was associated with a marked decrease in infarct area and an increase in left ventricular (LV) function after 3 and 6 months of follow-up.[17] To confirm these results and validate this promising new therapy for MI, we established a clinical trial involving 20 patients for comparing the safety and bioefficacy of autologous BMC transplantation. All 20 patients underwent standard therapy, and 10 patients received additional intracoronary cell transplantation. All 20 patients were followed up for 3 months.

Methods

Patient Population

All 20 patients had suffered transmural infarction according to World Health Organization criteria with the involvement of the left anterior descending coronary artery (n=4), left circumflex coronary artery (n=3), or right coronary artery (n=13). Mean duration of infarct pain was 12±10 hours before invasive diagnostics and therapy. Patients had to be <70 years old and were excluded if one of the following criteria were met: screening >72 hours after infarction, cardiac shock, severe comorbidity, alcohol or drug dependency, or excessive travel distance to the study center.

After right and left heart catheterization, coronary angiography, and left ventriculography, mechanical treatment was initiated with recanalization of the infarct-related artery by balloon angioplasty (n=20) and subsequent stent implantation (n=19). All patients were monitored in our intensive care unit, and no arrhythmogenic events or hemodynamic impairments were recorded in either patient group.

All 20 patients were briefed in detail about the procedure of BMC transplantation. Informed consent was obtained from 10 patients, who formed the cell therapy group, whereas 10 patients who refused additional cell therapy served as controls. The local ethics committee of the Heinrich-Heine-University, Düsseldorf, approved the study protocol. All procedures conformed to institutional guidelines.

Before taking part in rehabilitation programs, all patients left the hospital with standard medication consisting of acetylsalicylic acid, an ACE inhibitor, a β-blocker, and a statin.

Bone Marrow Aspiration, Isolation, and Cultivation

Seven (±2) days after acute coronary angiography, bone marrow (~40 mL) was aspirated under local anesthesia from ilium of cell therapy patients (n=10). Mononuclear BMCs were isolated by Ficoll density separation on Lymphocyte Separation Medium (BioWhittaker) before the erythrocytes were lysed with H₂O. For overnight

cultivation, 1×10⁶ BMCs/mL were placed in Teflon bags (Vuelife, Cell Genix) and cultivated in X-Vivo 15 Medium (BioWhittaker) supplemented with 2% heat-inactivated autologous plasma. The next day, BMCs were harvested and washed 3 times with heparinized saline before final resuspension in heparinized saline. Viability was 93±3%. Heparinization and filtration (cell strainer, FALCON) was carried out to prevent cell clotting and microembolization during intracoronary transplantation. The mean number of mononuclear cells harvested after overnight culture was 2.8×10⁷; this consisted of 0.65±0.4% AC133-positive cells and 2.1±0.28% CD34-positive cells. All microbiological tests of the clinically used cell preparations proved negative. As a viability and quality ex vivo control, 1×10³ cells grown in H5100 medium (Stem Cell Technology) were found to be able to generate mesenchymal cells in culture.

Intracoronary Transplantation of BMCs

Five to nine days after onset of acute infarction, cells were directly transplanted into the infarcted zone (Figure 1). This was accomplished with the use of a balloon catheter, which was placed within the infarct-related artery. After exact positioning of the balloon at the site of the former infarct-vessel occlusion, percutaneous transluminal coronary angioplasty (PTCA) was performed 6 to 7 times for 2 to 4 minutes each. During this time, intracoronary cell transplantation via the balloon catheter was performed, using 6 to 7 fractional high-pressure infusions of 2 to 3 mL cell suspension, each of which contained 1.5 to 4×10⁶ mononuclear cells. PTCA thoroughly prevented the backflow of cells and at the same time produced a stop-flow beyond the site of the balloon inflation to facilitate high-pressure infusion of cells into the infarcted zone. Thus, prolonged contact time for cellular migration was allowed.[18]

Functional Assessment of Hemodynamics

After 3 months, all 20 patients were followed up by left heart catheterization, left ventriculography, and coronary angiography. Ejection fraction, infarct region, and regional wall movement of the infarcted zone during ejection were determined by left ventriculography. Ejection fraction was measured with Quantcor software (Siemens). To quantify infarction wall movement velocity, 5 axes were placed perpendicular to the long axis in the main akinetic or dyskinetic segment of the ventricular wall. Relative systolic and diastolic lengths were measured, and the mean difference was divided by the systolic duration (in seconds). To quantify the infarct region, the centerline method according to Sheehan was used.[19] All hemodynamic investigations were obtained by two independent observers.

In the cell therapy group before and 3 months after cell transplantation, additional examinations for measuring hemodynamics and myocardial perfusion included dobutamine stress echocardiography, radionuclide ventriculography, catheterization of the right heart, and

TABLE 1. Baseline Characteristics of the Patients

Clinical Data	Cell Therapy	Standard Therapy	P
Characteristics			
No. of patients	10	10	...
Age, y	49±10	50±6	NS
Sex	Male	Male	...
Onset of infarction before angioplasty, h	10±8	13±11	NS
Coronary angiography			
No. of diseased vessels	1.7±0.9	2.1±0.7	NS
No. of patients with LAD/LCX/RCA as the affected vessel	4/1/5	0/2/8	...
No. of patients with stent implantation	9	10	...
Laboratory parameters			
Creatinine kinase, U/L	1138±1170	1308±1187	NS
Creatinine kinase-MB, U/L	106±72	124±92	NS
Bone marrow puncture after angioplasty, d	7±2	...	...
Mononuclear bone marrow cells, n (×10⁷)	2.8±2.2	...	...

Values are mean±SD or number of patients.
NS indicates not significant; LAD, left anterior descending coronary artery; LCX, left circumflex coronary artery; and RCA, right coronary artery.

stress-redistribution-reinjection ^{201}thallium scintigraphy. The contractility index P_{sys}/ESV was calculated by dividing LV systolic pressure (P_{sys}) by end-systolic volume (ESV). Perfusion defect was calculated by scintigraphic bull's-eye technique. Each examination was performed according to standard protocols.

There were no complications or side effects determined in any patient throughout the diagnostic or therapeutic procedure or within the 3-month follow-up period.

Statistical Analysis

All data are presented as mean±SD. Statistical significance was accepted when P was <0.05. Discrete variables were compared as rates, and comparisons were made by χ^2 analysis. Intra-individual comparison of baseline versus follow-up continuous variables was performed with a paired t test. Comparison of nonparametric data between the two groups was performed with Wilcoxon test and Mann-Whitney test. Statistical analysis was performed with SPSS for Windows (version 10.1).

Results

Clinical data between the two groups did not differ significantly. The range of creatinine kinase levels was slightly but not significantly higher in the standard therapy group than it was in the cell therapy group (Table 1).

Comparison of the 2 groups 3 months after cell or standard therapy showed several significant differences in LV dynamics, according to the global and regional analysis of left ventriculogram. The infarct region as a percentage of hypokinetic, akinetic, or dyskinetic segments of the circumference of the left ventricle decreased significantly in the cell therapy group (from 30±13 to 12±7%, P=0.005). It was also significantly smaller compared with the standard therapy group after 3 months (P=0.04). Within the standard therapy group, only a statistically nonsignificant decrease from 25±8 to 20±11% could be seen. Wall movement velocity over the infarct region rose significantly in the cell therapy group (from 2.0±1.1 to 4.0±2.6 cm/s, P=0.028) but not in the standard therapy group (from 1.8±1.3 to 2.3±1.6 cm/s, P=NS). No significant difference was observed between the

two groups. Ejection fraction increased in both groups, albeit nonsignificantly (from 57±8 to 62±10% in the cell therapy group and from 60±7 to 64±7% in the standard therapy group) (Table 2).

Further significant improvement could also be seen on additional analysis of the cell therapy group alone. Perfusion defect was considerably decreased by 26% in the cell therapy group (from 174±99 to 128±71 cm³, P=0.016, assessed by ^{201}thallium scintigraphy) (Figure 2). Parallel to the reduction in perfusion defect, improvement (Table 3) could also be seen in:

(1) Cardiac function, as revealed by increase in stroke volume index (from 49±7 to 56±7 mL/m², P=0.010) and ejection fraction (from 51±14 to 53±13%, P=NS).

(2) Cardiac geometry, as shown by decreases in both end-diastolic (from 158±20 to 143±30 mL, P=NS) and end-systolic volume (from 82±26 to 67±21 mL, P=0.011). Radionuclide ventriculography was used to acquire the data.

(3) Contractility as evaluated by an increase in the velocity of circumferential fiber shortening (from 20.5±4.2 to 24.4±7.7 mm/s, P=NS, assessed by stress echocardiography) and by a marked increase in the ratio of systolic pressure to end-systolic volume (from 1.81±1.44 to 2.27±1.72 mm Hg/mL, P=0.005).

Discussion

The present report describes the first clinical trial of intracoronary, autologous, mononuclear BMC transplantation for improving heart function and myocardial perfusion in patients after acute MI. The results demonstrate that transplanted autologous BMCs may lead to repair of infarcted tissue when applied during the immediate postinfarction period. These results also show that the intracoronary approach of BMC transplantation seems to represent a novel

TABLE 2. Comparison of Cell Therapy and Standard Therapy Groups

	Cell Therapy	Standard Therapy	P
No. of patients	10	10	...
Infarct region as functional defect			
Hypokinetic, akinetic, or dyskinetic region at 0 mo, %	30±13	25±8	NS
Hypokinetic, akinetic, or dyskinetic region at 3 mo, %	12±7	20±11	0.04
P	0.005	NS	...
Contractility indices			
Infarction wall movement velocity at 0 mo, cm/s	2.0±1.1	1.8±1.3	NS
Infarction wall movement velocity at 3 mo, cm/s	4.0±2.6	2.3±1.6	NS
P	0.028	NS	...
Hemodynamic data			
LV ejection fraction at 0 mo, %	57±8	60±7	NS
LV ejection fraction at 3 mo, %	62±10	64±7	NS
P	NS	NS	...

NS indicates not significant; 0 mo, zero months, which means the time of infarction; 3 mo, 3 months, which means the time of the follow-up examinations. All data were obtained according to analysis of left ventriculogram.

and effective therapeutic procedure for concentrating and/or depositing infused cells within the region of interest.

Neogenesis of both cardiomyocytes and coronary capillaries with some functional improvement has been shown recently by several investigators using bone marrow–derived cells in experimental infarction.[11-14,18,20-23] Moreover, transendothelial migration from the coronary capillaries and incorporation of cells into heart muscle has been observed experimentally.[3,13,24-26] Until now, clinical data only existed for the cell therapy of surgically treated chronic ischemic heart disease.[15,16] Our aim was to transform the encouraging results from animal models to a safe clinical setting. The most crucial questions we had to address while designing and

realizing this trial were: (1) What cell population should we deliver? (2) Which application method is the most efficient? (3) When should the cells be transplanted?

In recent years, several laboratories have shown that environmentally dictated changes of fate (transdetermination) are not restricted to stem cells but may also involve progenitor cells at different steps of a given differentiation pathway (transdifferentiation). Moreover, mesenchymal stem cells may represent an ideal cell source for treating different diseases.[27] Adult, mononuclear BMCs contain such stem and progenitor cells (≤1%), eg, mesodermal progenitor cells, hematopoietic progenitor cells, and endothelial progenitor cells. In several animal infarction models it has been shown that: (1) Bone marrow hemangioblasts contribute to the formation of new vessels; (2) bone marrow hematopoietic stem cells differentiate into cardiomyocytes, endothelium,

Figure 2. Improved myocardial perfusion of infarcted anterior wall 3 months after intracoronary cell transplantation subsequent to an acute anterior wall infarction detected by [201]thallium scintigraphy. The images on the left (A, D, sagittal) and in the middle (B, E) show the long axis, whereas those on the right (C, F, frontal) show the short axis of the heart. Initially the anterior wall, with green-colored apical and anterior regions, had reduced myocardial perfusion (A, B, C). Three months after cell transplantation the same anterior wall, now yellow in color, revealed a significant improvement in myocardial perfusion (D, E, F). All illustrations depict the exercise phase.

TABLE 3. Cardiac Function Analysis at 3-Month Follow-Up

	Before Cell Therapy	3 Months After Cell Therapy	P
No. of patients	10	10	...
Hemodynamic data			
LV ejection fraction, %	51±14	53±13	NS
Stroke volume index, mL/m²	49±7	56±7	0.010
Cardiac geometry			
LV end-diastolic volume, mL	158±20	143±30	NS
LV end-systolic volume, mL	82±26	67±21	0.011
Contractility indices			
Circumferential fiber shortening, mm/s	20.5±4.2	24.4±7.7	NS
P_{max}/ESV, mm Hg/mL	1.81±1.44	2.27±1.72	0.005
Infarct region as perfusion defect			
[201]Thallium scintigraphy, cm²	174±99	128±71	0.016

NS indicates not significant.

and smooth muscle cells[9-13]; (3) BMCs give rise to mesodermal progenitor cells that differentiate to endothelial cells[18]; and (4) endothelial progenitors can transdifferentiate into beating cardiomyocytes.[19] Thus, several different fractions of mononuclear BMCs may contribute to the regeneration of necrotic myocardium and vessels. In order to utilize this large and perhaps heterogeneous regenerative potential, we decided to use all mononuclear cells from the bone marrow aspirate as a whole, rather than a subpopulation. No further expansion was performed because experimental data have revealed a dramatic decline in the homing capacity of in vitro amplified hematopoietic stem or progenitor cells.[30]

The second question was how to deliver the cells most efficiently. When given intravenously, only a very small fraction of infused cells can reach the infarct region after the following injection: assuming a normal coronary blood flow of 80 mL/min per 100 g of LV weight, a quantity of 160 mL per left ventricle (assuming a regular LV mass of ~200 g) will flow per minute.[31,32] This corresponds to only about 3% of cardiac output (assuming a cardiac output of 5000 mL/min).[31] Therefore, intravenous application would require many circulation passages to enable infused cells to come into contact with the infarct-related artery. Throughout this long circulation and recirculation time, homing of cells to other organs could considerably reduce the numbers of cells dedicated to cell repair in the infarcted zone. Thus, supplying the entire complement of cells by intracoronary administration obviously seems to be advantageous for the tissue repair of infarcted heart muscle and may also be superior to intraventricular injection,[33] because all cells are able to flow through the infarcted and peri-infarcted tissue during the immediate first passage. Accordingly, by this intracoronary procedure the infarct tissue and the peri-infarct zone can be enriched with the maximum available amount of cells at all times.

As stem cells differentiate into more mature types of progenitor cells, it is thought that a special microenvironment in so-called niches regulates cell activity by providing specific combinations of cytokines and by establishing direct cellular contact. For successful long-term engraftment, at least some stem cells have to reach their niches, a process referred to as homing. Mouse experiments have shown that significant numbers of BMCs appear in liver, spleen, and bone marrow after intravenous injection.[34] To offer the BMCs the best chance of finding their niche within the myocardium, a selective intracoronary delivery route was chosen. Presumably, therefore, fewer cells were lost by extraction toward organs of secondary interest by this first pass–like effect. To facilitate transendothelial passage and migration into the infarcted zone, cells were infused by high-pressure injection directly into the necrotic area, and the balloon was kept inflated for 2 to 3 minutes; the cells were not washed away immediately under these conditions.

The time point for delivery was chosen as 7 to 8 days after infarction onset for the following reasons:

(1) In dogs, infarcted territory becomes rich in capillaries and contains enlarged, pericyte-poor "mother vessels" and endothelial bridges 7 days after myocardial ischemia and reperfusion. Twenty-eight days later, a significant muscular vessel wall has already formed.[35] Thus, with such timing, cells may be able to reach the worst

damaged parts and at the same time salvage tissue. Transendothelial cell migration may also be enhanced because an adequate muscular coat is not yet formed.

(2) Until now, only one animal study has attempted to determine the optimum time for cardiomyocyte transplantation to maximize myocardial function after LV injury. Adult rat hearts were cryoinjured and fetal rat cardiomyocytes were transplanted immediately, 2 weeks later, and 4 weeks later. The authors discussed the inflammatory process, which is strongest in the first days after infarction, as being responsible for the negative results after immediate cell transplantation, and they assumed that the best results seen after 2 weeks may have been due to transplantation before scar expansion.[36] Until now, however, no systematic experiments have been performed with BMCs to correlate the results of transplantation with the length of such a time delay.

(3) Another important variable is the inflammatory response in MI, which seems to be a superbly orchestrated interaction of cells, cytokines, growth factors and extracellular matrix proteins mediating myocardial repair. In the first 48 hours, debridement and formation of a fibrin-based provisional matrix predominates before a healing phase ensues.[37-40] Moreover, vascular endothelial growth factor is at its peak concentration 7 days after MI, and the decline of adhesion molecules (intercellular adhesion molecules, vascular cell adhesion molecules) does not take place before days 3 to 4 after MI. We assumed that transplantation of mononuclear BMCs within the "hot" phase of post-MI inflammation might lead them to take part in the inflammation cascade rather than the formation of functional myocardium and vessels.

Taking all of this into account, we can conclude that cell transplantation within the first 5 days after acute infarction is not possible for logistical reasons and is not advisable because of the inflammatory process. On the other hand, transplantation 2 weeks after infarction scar formation seems to reduce the benefit of cell transplantation. Although the ideal time point for transplantation remains to be defined, it is most likely between days 7 and 14 after the onset of MI, as in the present study.

This trial was designed as a phase I safety and feasibility trial, meaning that no control group is necessarily required. However, to validate the results, we correlated them with those obtained from 10 patients who refused to get additional cell therapy and thus received standard therapy alone. We are aware of the fact that such a comparison does not reach the power of a randomly allocated, blinded control group. However, the significant improvement with regard to infarct region, hemodynamics (stroke volume index), cardiac geometry (LV end-systolic volume), and contractility (P_{in}/ESV and infarction wall movement velocity) did confirm a positive effect of the additional cell therapy because the changes observed in the standard therapy group failed to reach significance.

Another important factor for interpreting the results is time interval between onset of symptoms and revascularization of the infarct-related artery by angioplasty; this represents a crucial determinant of LV recovery. For patients with acute MI, it has

been shown that if the time interval is >4 hours, no significant changes in ejection fraction, regional wall motion, or ESV are observed after 6-month follow-up by echocardiography and angiography.[41] None of our 20 patients was treated by angioplasty within 4 hours after onset of symptoms. Our average time interval was 12 ± 10 hours. Thus, PTCA-induced improvement of LV function can be nearly excluded; indeed, the only mild and nonsignificant changes within the standard therapy group are consistent with the above-mentioned data.[41] In contrast, the cell therapy group showed considerable and significant improvement in the same parameters, which may be attributed to BMC-mediated coronary angioneogenesis and cardiomyoneogenesis.

These results show that transplantation of autologous BMCs, as well as the intracoronary approach, represent a novel and effective therapeutic procedure for the repair of infarcted myocardium. For this method of therapy, no ethical problems exist, and no side effects were observed at any point of time. The therapeutic benefit for the patient's heart seems to prevail. However, further experimental studies, controlled prospective clinical trials, and variations of cell preparations are required to define the role of this new approach for the therapy of acute MI in humans.

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EVIDENCE APPENDIX

ITEM NO. 5

Second Supplemental Declaration of Dr. Andrew E. Lorincz
cited by Appellant as Exhibit D
in the Response filed June 26, 2006

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Re Application of: James P. Elia   )
) Group Art Unit: 1646
Serial No.: 09/064,000   )
) Examiner: Elizabeth C. Kemmerer, Ph.D.
Filed: April 21, 1998   )
)
For: METHOD FOR GROWTH)
  OF SOFT TISSUE   )

SECOND SUPPLEMENTAL DECLARATION
OF ANDREW E. LORINCZ, M.D.

I Andrew E. Lorincz declare as follows:

1. I reside at 16135 NW 243rd Way, High Springs, Florida 32643-3813.

2. My Curriculum Vitae is attached as Exhibit A to my Declaration of February 12, 2001. Paragraph 4 of my Declaration and my Supplemental Declaration of November 8, 2004 provide additional information regarding my background and experience.

3. I have read and understood the disclosures of the above-referenced patent application at page 20, line 10 through page 21, line 15; at page 37, lines 19-25; at page 44, line 19 through page 46, line 16; and at page 47, line 22 through page 48, line 15. Such disclosures are the same as I read and understood in my

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previous Declaration. A copy of such disclosures is attached hereto as Second Supplemental Declaration Exhibit A.

I have also read and understood additional disclosures of the above-referenced patent application at page 33, lines 8-10; page 40, line 20 through page 43 line 3; page 44, lines 12 and 13; page 48, lines 13-15; page 53, line 1 through page 56, line 25; and page 62, lines 1-10. A copy of such additional disclosures is attached hereto as Second Supplemental Declaration Exhibit B.

4. I note that the disclosures referenced in above Paragraph 3 relate to using a growth factor for promoting the growth of soft tissue, and more specifically, to a method of using a cell, such as a stem cell, to grow soft tissue, such as an artery.

5. I have read and understood the claims set forth in the attached Second Supplemental Declaration Exhibit C and have been informed that such claims will be concurrently presented in the above-referenced patent application with this Second Supplemental Declaration.

6. Based upon above Paragraphs 3-5, it is my opinion that introducing a growth factor, including cells, and more specifically, stem cells, in the body of a human patient will predictably result in the growth of soft tissue, such as an artery, which will integrate itself into pre-existing tissue of the body thereby forming a unified whole.

7. Based upon above Paragraphs 3-5, it is my opinion that one skilled in the medical arts, armed with the knowledge in such paragraphs, would be able to practice the method set forth in Exhibit C without need for resorting to undue experimentation.

8. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: _5 June 2006_

Andrew E. Lorincz, M.D.
Andrew E. Lorincz, M.D.

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SECOND SUPPLEMENTAL DECLARATION

EXHIBIT A

DISCLOSURES

EXHIBIT A

DISCLOSURES
APPLICATION SERIAL NO. 09/064,000

PAGE 20, LINE 10 – PAGE 21, LINE 15

Growth factors can be utilized to induce the growth of "hard tissue" or bone and "soft tissues" like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basis (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

- A1 -

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor an also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which an often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 37, LINES 19-25

Multifactorial and nonspecific cells (such as stem cells and germinal cells) can provide the necessary in vivo and in vitro cascade of genetic material once an implanted master control gene's transcription has been activated. Likewise, any host cell, clone cell, cultured cell, or cell would work. Genetic switches (such as the insect hormone ecdysone) can be used to control genes inserted into humans and animals. These gene switches can also be used in cultured cells or other cells. Gene switches govern whether a gene is on or off making possible precise time of gene activity.

PAGE 44, LINE 19 – PAGE 46, LINE 16

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in connection with any of the implant techniques of the invention, it is understood that a cell nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated (taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which

promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth factors (or other genetic material) are placed. Once the new muscle and blood vessels have

grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

PAGE 47, LINE 22 – PAGE 48, LINE 15

Organs and/or tissues can be formed utilizing the patient's own cells. For example, a skin cell(s) is removed from the intraoral lining of a cheek. The cell is genetically screened to identify DNA damage or other structural and/or functional problems. Any existing prior art genetic screening technique can be utilized. Such methods can utilize lasers, DNA probes, PCR, or any other suitable device. If the cell is damaged, a healthy undamaged cell is, if possible, identified and selected. If a healthy cell can not [sic] be obtained, the damaged cell can be repaired by excision, alkylation, transition or any other desired method. A growth factor(s) is added to the cell to facilitate dedifferentiation and then redifferentiation and morphogenesis into an organ or function specific tissue. Any machine known in the art can be used to check the genetic fitness of the organ and its stage of morphogenesis. A cell nutrient culture may or may not be utilized depending on the desired functional outcome (i.e., growth of an artery, of pancreatic Islet cells, of a heart, etc.) or other circumstances. Replantation can occur at any appropriate stage of morphogenesis. The foregoing can be repeated without the patient's own

cells if universal donor cells such a [sic] germinal cells are utilized. Germinal cells do not require a dedifferentiation. They simply differentiate into desired tissues or organs when properly stimulated. Similarly, the DNA utilized in the foregoing procedure can come from the patient or from any desired source.

During reimplantation one of the patient's own cells is returned to the patient. During implantation, a cell not originally obtained from the patient is inserted on or in the patient.

In the example above, if germinal cells (and in some case, stem cells) are utilized a direct differentiation and morphogenesis into an organ can occur in vivo, ex vivo, or in vitro.

SECOND SUPPLEMENTAL DECLARATION

EXHIBIT B

DISCLOSURES

EXHIBIT B

DISCLOSURES
APPLICATION SERIAL NO. 09/064,000

PAGE 33, LINES 8-10

Morphogenesis or morphogenetics is the origin and evolution of morphological characters and is the growth and differentiation of cells and tissues during development.

PAGE 40, LINE 20 – PAGE 43, LINE 3

EXAMPLE 11

MSX-1 and MSX-2 are the homeobox genes that control the generation and growth of a tooth. A sample of skin tissue is removed from the patient and the MSX-1 and MXS-2 homeobox gene(s) are removed from skin tissue cells. The genes are stored in an appropriate nutrient culture medium.

BMP-2 and BMP-4 growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

MXS-1 and MXS-2 transcription factors are obtained which will initiate the expression of the MXS-1 and MXS-2 homeobox genes.

The MXS-1 and MXS-2 transcription factors, BMP-2 and BMP-4 bone morphogenic proteins, and MXS-1 and MXS-2 genes are added to the nutrient culture medium along with the living stem cells.

EXAMPLE 12

Example 11 is repeated except that the transcription factors bind to a receptor complex in the stem cell nucleus.

EXAMPLE 13

Example 11 is repeated except that the MXS-1 and MXS-2 transcription factors are not utilized. The transcription of the MXS-1 and MXS-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 14

Example 13 is repeated except that the stem cells are starved and the transcription of the MXS-1 and MXS-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 15

WT-1 and PAX genes are obtained from a sample of skin tissue is removed from the patient. The genes are stored in an appropriate nutrient culture medium. PAX genes produce PAX-2 and other transcription factors.

BMP-7 and other kidney related BMP growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees.

The temperature of the culture medium can be varied as desired but ordinarily is between 40 and 102 degrees F.

The WT-1 and PAX genes, and BMP-7 and other kidney BMPS are added to the nutrient culture medium along with the living stem cells.

A primitive kidney germ is produced. The kidney germ is transplanted in the patient's body near a large artery. As the kidney grows, its blood supply will be derived from the artery.

EXAMPLE 16

The Aniridia gene is obtained from a sample of skin tissue is removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

The Aniridia transcription factor and growth factors and the Aniridia gene are added to the nutrient culture medium along with the living stem cells.

A primitive eye germ is produced. The kidney germ is transplanted in the patient's body near the optic nerve. As the kidney grows, its blood supply will be derived from nearby arteries.

EXAMPLE 17

The Aniridia gene is obtained from a sample of skin tissue is removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained and added to the nutrient culture medium.

An eye germ develops. A branch of the nearby maxillary artery is translocated to a position adjacent the eye germ to promote the development of the eye germ. The eye germ matures into an eye which receives its blood supply from the maxillary artery.

The term "cell nutrient culture" as used herein can include any or any combination of the following: the extracellular matrix; conventional cell culture nutrients; and/or, a cell nutrient such as a vitamin. As such, the cell nutrient culture can be two-dimensional, three dimensional, or simply a nutrient, and is useful in promoting the processes of cellular dedifferentiation, redifferentiation, differentiation, growth, and development.

PAGE 44, LINES 12– 13

An organ, as used herein, consists of two or more kinds of tissues joined into one structure that has a certain task.

PAGE 48, LINES 13– 15

In the example above, if germinal cells (and in some cases, stem cells) are utilized a direct differentiation and morphogenesis into an organ can occur in vivo, ex vivo, or in vitro.

EXAMPLE 18

A 36 year old Caucasian male experiences pain in his left leg. A medical examination reveals a damaged one inch long section of a large artery in his left leg. The examination also reveals that this damaged section of the artery is nearly completely clogged with plaque and that the wall of the artery is weakened. The weakening in the arterial wall makes attempting to clean out the artery risky and also makes it risky to attempt to insert a stent in the artery.

Recombinant cDNA encoded to combine with a cell ribosome to produce the human growth factor VEGF is assembled into a eukaryotic expression plasmid. The recombinant cDNA is from cDNA libraries prepared from HL60 leukemia cells and is known to cause the growth of arteries. The plasmid is maintained at a room temperature of 76 degrees F.

The clones are placed in 1.0 milliliters of a normal saline carrier solution at a room temperature of 76 degrees F to produce an genetic carrier solution. The genetic carrier solution contains about 250 ug of the cDNA clones. A nutrient culture can, if desired, be utilized in conjunction with or in place of the saline carrier. Each clone is identical. If desired, only a single clone can be inserted in the normal saline carrier solution. The saline carrier solution comprises 0.09% by weight sodium chloride in water. A saline carrier solution is selected because it will not harm the DNA clone.

Two sites are selected for injection of the genetic carrier solution. While the selection of sites can vary as desired, the sites are selected at the lower end (the end nearest the left foot of the patient) of the damaged section of the artery so that the new arterial section grown can, if necessary, be used to take the place of the damaged section of the artery in the event the damaged section is removed.

The first site is on the exterior wall of the artery on one side of the lower end of the damaged section of the artery. A containment system is placed at the first site.

The second site is inside the wall of the artery on the other side of the lower end of the artery.

The genetic carrier solution is heated to a temperature of 98.6 degrees F. 0.25 milliliters of the genetic carrier solution is injected into the containment system at the first site. 0.25 milliliters of the genetic carrier solution is injected at the second site inside the wall of the artery. Care is taken to slowly inject the genetic carrier solution to avoid entry of the solution into the artery such that blood stream will carry away the cDNA in the solution.

After two weeks, an MRI is taken which shows the patient's leg artery. The MRI reveals new growth at the first and second sites.

After four weeks, another MRI is taken which shows the patient's leg artery. The MRI shows that (1) at the first site a new artery is growing adjacent the patient's original leg artery, and (2) at the second site a new section of artery is growing integral with the original artery, i.e., at the second site the new section of artery is lengthening the original artery, much like inserting a new section of hose in a garden hose concentric with the longitudinal axis of the garden hose lengthens the garden hose.

After about eight to twelve weeks, another MRI is taken which shows that the new artery growing adjacent the patient's original artery has grown to a length of about one inch and has integrated itself at each of its ends with the original artery such that blood flows through the new section of artery. The MRI also shows that the new artery at the second site has grown to a length of one-half inch.

In any of the examples of the practice of the invention included herein, cell nutrient culture can be included with the gene, the growth factor, the extracellular matrix, or the environmental factors.

In any of the examples of the practice of the invention included herein, the concept of gene redundancy can be applied. For example, the Examples 1 to 14 concerning a tooth list the genes MSX-1 and MSX-2. These genes differ by only two base pairs. Either gene alone may be sufficient. A further example of redundancy occurs in growth factors. Looking at the Examples 10 to 14, BMP4 or BMP2 alone may be sufficient. Redundancy can also be utilized in connection with transcription factors, extracellular matrices, environmental factors, cell nutrient cultures, physiological nutrient cultures, vectors, promotors, etc.

One embodiment of the invention inserts genetic material (gene, growth factor, ECM, etc.) into the body to induce the formation of an organ. Similar inducing materials inserted ex vivo into or onto a living cell in an appropriate physiological nurturing environment will also induce the growth of an organ. The VCSEL laser allows early detection in a living cell of a morphogenic change indicating that organ formation has been initiated. With properly timed transplantation, organ growth completes itself.

During the ex vivo application of the invention, a gene and/or growth factor is inserted into a cell or a group of cells; an ECM or environmental factor(s) are placed around and in contact with a cell or group of cells; or, genetic material is inserted into a subunit of a cell to induce organ growth. An example of a subunit of a cell is an enucleated cell or a comparable artificially produced environment. In in vivo or ex vivo embodiments of the invention to induce the growth of an organ, the genes, growth factors, or other genetic material, as well as the environmental factors or cells utilized, can come from any desired source.

EXAMPLE 19

Genetically produced materials are inserted in the body to cause the body to grow, reproduce, and replace in vivo a clogged artery in the heart. This is an example of site-specific gene expression. A plasmid expression vector containing an enhancer/promoter is utilized to aid in the transfer of the gene into muscle cells. The enhancer is utilized to drive the specific expression of the transcriptional activator. After the enhancer drives the expression of the transcriptional activator, the transcriptional activator transactivates the muscle/artery genes. Saline is used as a carrier. Cardiac muscle can take up naked DNA injected intramuscularly. Injecting plasmid DNA into cardiac (or skeletal) muscle results in expression of the transgene in cardiac myocytes for several weeks or longer.

Readily available off-the-shelf (RAOTS) cDNA clones for recombinant human VEGF165, isolated from cDNA libraries prepared from HL60 leukemia cells, are assembled in a RAOTS expression plasmid utilizing 736 bp CMV promoter/enhancer to drive VEGF expression. Other RAOTS promoters can be utilized to drive VEGF expression for longer periods of time. Other RAOTS recombinant clones of angiogenic growth factors other than VEGF can be utilized, for example, fibroblast growth factor family, endothelial cell growth factor, etc. Downstream from the VEGF cDNA is an SV40 polyadenylation sequence. These fragments occur in the RAOTS pUC118 vector, which includes an Escherichia coli origin of replication and the Beta lactamase gene for ampicillin resistance.

The RAOTS construct is placed into a RAOTS 3 ml syringe with neutral pH physiologic saline at room temperature (or body temperature of about 37 degrees C). The syringe has a RAOTS 27 gauge needle.

Access to the cardiac muscle is gained by open heart surgery, endoscopic surgery, direction injection of the needle without incision, or by any other desired means. The cardiac muscle immediately adjacent a clogged artery is slowly injected with the RAOTS construct during a five second time period. Injection is slow to avoid leakage through the external covering of muscle cells. About 0.5 ml to 1.0 ml (milliliter) of fluid is injected containing approximately 500 ug phVEGF165 in saline (N=18). The readily available off-the-shelf cDNA clones cause vascular growth which automatically integrates itself with the cardiac muscle. Anatomic evidence of collateral artery formation is observed by the 30th day following injection to the RAOTS construct. One end of the artery integrates itself in the heart wall to receive blood from the heart. The other end of the artery branches into increasing smaller blood vessels to distribute blood into the heart muscle. Once the growth of the new artery is completed, the new artery is left in place in the heart wall. Transplantation of the new artery is not required.

Blood flow through the new artery is calculated in a number of ways. For example, Doppler-derived flow can be determined by electromagnetic flowmeters (using for example, a Doppler Flowmeter sold by Parks Medical Electronic of Aloha, Oregon) both in vitro and in vivo. RAOTS external ultrasound gives a semiquantitative analysis of arterial flow. Also, RAOTS angiograms or any other readily available commercial devices can be utilized.

VEGF gene expression can be evaluated by readily available off-the-shelf polymerase chain reaction (PCR) techniques.

If controls are desired, the plasmid pGSVLacZ containing a nuclear targeted Beta-galactosidase sequence coupled to the simian virus 40 early promoter can be used. To evaluate efficiency, a promoter-matched reporter plasmid, pCMV Beta (available from Clontech of Palo

Alto, California), which encodes Beta-galactosidase under control of CMV promoter/enhancer can be utilized. Other RAOTS products can be utilized if desired.

EXAMPLE 20

A patient, a forty year old African-American female in good health, has been missing tooth number 24 for ten years. The space in her mouth in which her number 24 tooth originally resided is empty. All other teeth except tooth number 24 are present in the patient's mouth. The patient desires a new tooth in the empty "number 24" space in her mouth.

A full thickness mucoperiosteal flap surgery is utilized to expose the bone in the number 24 space. A slight tissue reflection into the number 23 tooth and number 25 tooth areas is carried out to insure adequate working conditions.

A Midwest Quietair handpiece (or other off-the-shelf handpiece) utilizing a #701XXL bur (Dentsply Midwest of Des Plaines, Illinois) (a #700, #557, #558, etc. bur can be utilized if desired) is used to excavate an implant opening or site in the bone. The implant opening is placed midway between the roots of the number 23 and number 25 teeth. The opening ends at a depth which is about fifteen millimeters and which approximates the depth of the apices of the roots of the number 23 and number 25 teeth. Care is taken not to perforate either the buccal or lingual wall of the bone. In addition, care is taken not to perforate or invade the periodontal ligament space of teeth numbers 23 and 25.

An interrupted drilling technique is utilized to avoid overheating the bone when the #701XXL bur is utilized to form the implant opening. During a drilling sequence, the drill is operated in five second increments and the handpiece is permitted to stall. Light pressure and a gentle downward stroke are utilized.

EXAMPLE 36

Example 18 is repeated except that the patient is a 55 year old Caucasian male, and the genetic carrier solution is injected into two sites in the coronary artery of the patient. The first site is on the exterior wall on one side of the artery. The second site is inside the wall of the artery on the other side of the artery. A section of the artery is damaged, is partially blocked, and has a weakened wall. The first and second sites are each below the damaged section of the artery. Similar results are obtained, i.e., a new section of artery grows integral with the original artery, and a new section of artery grows adjacent the original artery. The new section of artery has integrated itself at either end with the original artery so that blood flows through the new section of artery.

SECOND SUPPLEMENTAL DECLARATION

EXHIBIT C

CLAIMS

EXHIBIT C

CLAIMS
APPLICATION SERIAL NO. 09/064,000

Claim 382   A method for producing and integrating tissue consisting of a desired soft tissue at a selected site in a body of a human patient comprising:

(a) Placing cells in said body of said human patient;

(b) Forming a bud at said selected site in said body of said human patient; and

(c) Growing said desired soft tissue which integrates itself into said body of said human patient from said bud.

Claim 383   The method of claim 382, wherein said cells are multifactorial and non-specific.

Claim 384   The method of claim 383, wherein said cells comprise stem cells.

Claim 385   The method of claim 382 further comprising forming a new artery.

Claim 386   The method of claim 383 further comprising forming a new artery.

Claim 387   The method of claim 382, wherein said soft tissue comprises mesodermal tissue.

Claim 388   The method of claim 382, wherein said soft tissue comprises an artery.

Claim 389   The method of claim 382, wherein said cells comprise stem cells.

Claim 390   The method of claim 389, wherein said soft tissue comprises an artery.

Claim 391   The method of claim 382, wherein said cells comprise pluripotent cells.

Claim 392   The method of claim 391, wherein said soft tissue comprises an artery.

Claim 393   The method of claim 391, wherein said cells comprise stem cells.

Claim 394   The method of claim 393, wherein said stem cells are multifactorial and non-specific.

Claim 395   The method of claim 382, wherein said cells are injected into said body.

Claim 396   The method of claim 382, wherein said cells are locally placed into said body.

Claim 397   The method of claim 396, wherein said cells comprise stem cells.

Claim 398   The method of claim 396, wherein said cells are injected intramuscularly.

Claim 399   The method of claim 397, wherein said stem cells are injected intramuscularly.

Claim 400   The method of claim 388 further comprising determining blood flow through said new artery.

Claim 401   The method of claim 388 further comprising observing said new artery.

Claim 402   The method of claim 399, wherein said selected site comprises a leg of said patient.

EVIDENCE APPENDIX

ITEM NO. 6

Third Supplemental Declaration of Dr. Andrew E. Lorincz
cited by Appellant as Exhibit B
in the Response filed April 30, 2007

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Re Application of: James P. Elia)	
)	Group Art Unit: 1646
Serial No.: 09/064,000)	
)	Examiner: Elizabeth C. Kemmerer, Ph.D.
Filed: April 21, 1998)	
)	
For: METHOD FOR GROWTH)	
OF SOFT TISSUE)	

**THIRD SUPPLEMENTAL DECLARATION
OF ANDREW E. LORINCZ, M.D.**

I Andrew E. Lorincz declare as follows:

1.   I reside at 16135 NW 243rd Way, High Springs, Florida 32643-3813.

2.   My Curriculum Vitae is attached as Exhibit A to my Declaration of February 12, 2001.   Paragraph 4 of my Declaration and my Supplemental Declaration of November 8, 2004 provide additional information regarding my background and experience.

3.   I have read and understood the disclosures of the above-referenced patent application at page 20, line 10 through page 21, line 15; at page 37, lines 19-25; at page 44, line 19 through page 46, line 16; and at page 47, line 22 through page 48, line 15.   Such disclosures are the same as I read and understood in my

previous Declaration. A copy of such disclosures is attached hereto as Third

Supplemental Declaration Exhibit A.

I have also read and understood additional disclosures of the above-referenced

patent application at page 33, lines 8-10; page 40, line 20 through page 43 line 3;

page 44, lines 12 and 13; page 48, lines 13-15; page 53, line 1 through page 56,

line 25; and page 62, lines 1-10. A copy of such additional disclosures is

attached hereto as Third Supplemental Declaration Exhibit B.

4. I note that the disclosures referenced in above Paragraph 3 relate to using a

growth factor for promoting the growth of soft tissue, and more specifically, to a

method of using a cell, such as a stem cell, to grow soft tissue, such as an artery.

5. I have read and understood the claims set forth in the attached Third

Supplemental Declaration Exhibit C and have been informed that such claims

will be concurrently presented in the above-referenced patent application with

this Third Supplemental Declaration.

6. Based upon above Paragraphs 3-5, it is my opinion that introducing a growth

factor, including cells, and more specifically, stem cells, in the body of a human

patient will predictably result in the growth of soft tissue, such as an artery,

which will integrate itself into pre-existing tissue of the body thereby forming a

unified whole.

7.   Based upon above Paragraphs 3-5, it is my opinion that one skilled in the medical arts, armed with the knowledge in such paragraphs, would be able to practice the method set forth in Exhibit C without need for resorting to undue experimentation.

8.   Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 2 - 22 - 07

Andrew E. Lorincz, M.D.

THIRD SUPPLEMENTAL
DECLARATION

EXHIBIT A

DISCLOSURES

PAGE 20, LINE 10 – PAGE 21, LINE 15

Growth factors can be utilized to induce the growth of "hard tissue" or bone and "soft tissues" like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basis (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor an also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which an often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 37, LINES 19-25

Multifactorial and nonspecific cells (such as stem cells and germinal cells) can provide the necessary in vivo and in vitro cascade of genetic material once an implanted master control gene's transcription has been activated. Likewise, any host cell, clone cell, cultured cell, or cell would work. Genetic switches (such as the insect hormone ecdysone) can be used to control genes inserted into humans and animals. These gene switches can also be used in cultured cells or other cells. Gene switches govern whether a gene is on or off making possible precise time of gene activity.

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in connection with any of the implant techniques of the invention, it is understood that a cell nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated (taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which

promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth factors (or other genetic material) are placed. Once the new muscle and blood vessels have

grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

PAGE 47, LINE 22 – PAGE 48, LINE 15

Organs and/or tissues can be formed utilizing the patient's own cells. For example, a skin cell(s) is removed from the intraoral lining of a cheek. The cell is genetically screened to identify DNA damage or other structural and/or functional problems. Any existing prior art genetic screening technique can be utilized. Such methods can utilize lasers, DNA probes, PCR, or any other suitable device. If the cell is damaged, a healthy undamaged cell is, if possible, identified and selected. If a healthy cell can not [sic] be obtained, the damaged cell can be repaired by excision, alkylation, transition or any other desired method. A growth factor(s) is added to the cell to facilitate dedifferentiation and then redifferentiation and morphogenesis into an organ or function specific tissue. Any machine known in the art can be used to check the genetic fitness of the organ and its stage of morphogenesis. A cell nutrient culture may or may not be utilized depending on the desired functional outcome (i.e., growth of an artery, of pancreatic Islet cells, of a heart, etc.) or other circumstances. Replantation can occur at any appropriate stage of morphogenesis. The foregoing can be repeated without the patient's own

cells if universal donor cells such a [sic] germinal cells are utilized. Germinal cells do not require a dedifferentiation. They simply differentiate into desired tissues or organs when properly stimulated. Similarly, the DNA utilized in the foregoing procedure can come from the patient or from any desired source.

During reimplantation one of the patient's own cells is returned to the patient. During implantation, a cell not originally obtained from the patient is inserted on or in the patient.

In the example above, if germinal cells (and in some case, stem cells) are utilized a direct differentiation and morphogenesis into an organ can occur in vivo, ex vivo, or in vitro.

THIRD SUPPLEMENTAL DECLARATION

EXHIBIT B

DISCLOSURES

EXHIBIT B

DISCLOSURES
APPLICATION SERIAL NO. 09/064,000

PAGE 33, LINES 8-10

Morphogenesis or morphogenetics is the origin and evolution of morphological characters and is the growth and differentiation of cells and tissues during development.

PAGE 40, LINE 20 – PAGE 43, LINE 3

EXAMPLE 11

MSX-1 and MSX-2 are the homeobox genes that control the generation and growth of a tooth. A sample of skin tissue is removed from the patient and the MSX-1 and MXS-2 homeobox gene(s) are removed from skin tissue cells. The genes are stored in an appropriate nutrient culture medium.

BMP-2 and BMP-4 growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

MXS-1 and MXS-2 transcription factors are obtained which will initiate the expression of the MXS-1 and MXS-2 homeobox genes.

The MXS-1 and MXS-2 transcription factors, BMP-2 and BMP-4 bone morphogenic proteins, and MXS-1 and MXS-2 genes are added to the nutrient culture medium along with the living stem cells.

EXAMPLE 12

Example 11 is repeated except that the transcription factors bind to a receptor complex in the stem cell nucleus.

EXAMPLE 13

Example 11 is repeated except that the MXS-1 and MXS-2 transcription factors are not utilized. The transcription of the MXS-1 and MXS-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 14

Example 13 is repeated except that the stem cells are starved and the transcription of the MXS-1 and MXS-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 15

WT-1 and PAX genes are obtained from a sample of skin tissue is removed from the patient. The genes are stored in an appropriate nutrient culture medium. PAX genes produce PAX-2 and other transcription factors.

BMP-7 and other kidney related BMP growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees.

The temperature of the culture medium can be varied as desired but ordinarily is between 40 and 102 degrees F.

The WT-1 and PAX genes, and BMP-7 and other kidney BMPS are added to the nutrient culture medium along with the living stem cells.

A primitive kidney germ is produced. The kidney germ is transplanted in the patient's body near a large artery. As the kidney grows, its blood supply will be derived from the artery.

EXAMPLE 16

The Aniridia gene is obtained from a sample of skin tissue is removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

The Aniridia transcription factor and growth factors and the Aniridia gene are added to the nutrient culture medium along with the living stem cells.

A primitive eye germ is produced. The kidney germ is transplanted in the patient's body near the optic nerve. As the kidney grows, its blood supply will be derived from nearby arteries.

EXAMPLE 17

The Aniridia gene is obtained from a sample of skin tissue is removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained and added to the nutrient culture medium.

An eye germ develops. A branch of the nearby maxillary artery is translocated to a position adjacent the eye germ to promote the development of the eye germ. The eye germ matures into an eye which receives its blood supply from the maxillary artery.

The term "cell nutrient culture" as used herein can include any or any combination of the following: the extracellular matrix; conventional cell culture nutrients; and/or, a cell nutrient such as a vitamin. As such, the cell nutrient culture can be two-dimensional, three dimensional, or simply a nutrient, and is useful in promoting the processes of cellular dedifferentiation, redifferentiation, differentiation, growth, and development.

PAGE 44, LINES 12– 13

An organ, as used herein, consists of two or more kinds of tissues joined into one structure that has a certain task.

PAGE 48, LINES 13– 15

In the example above, if germinal cells (and in some cases, stem cells) are utilized a direct differentiation and morphogenesis into an organ can occur in vivo, ex vivo, or in vitro.

EXAMPLE 18

A 36 year old Caucasian male experiences pain in his left leg. A medical examination reveals a damaged one inch long section of a large artery in his left leg. The examination also reveals that this damaged section of the artery is nearly completely clogged with plaque and that the wall of the artery is weakened. The weakening in the arterial wall makes attempting to clean out the artery risky and also makes it risky to attempt to insert a stent in the artery.

Recombinant cDNA encoded to combine with a cell ribosome to produce the human growth factor VEGF is assembled into a eukaryotic expression plasmid. The recombinant cDNA is from cDNA libraries prepared from HL60 leukemia cells and is known to cause the growth of arteries. The plasmid is maintained at a room temperature of 76 degrees F.

The clones are placed in 1.0 milliliters of a normal saline carrier solution at a room temperature of 76 degrees F to produce an genetic carrier solution. The genetic carrier solution contains about 250 ug of the cDNA clones. A nutrient culture can, if desired, be utilized in conjunction with or in place of the saline carrier. Each clone is identical. If desired, only a single clone can be inserted in the normal saline carrier solution. The saline carrier solution comprises 0.09% by weight sodium chloride in water. A saline carrier solution is selected because it will not harm the DNA clone.

Two sites are selected for injection of the genetic carrier solution. While the selection of sites can vary as desired, the sites are selected at the lower end (the end nearest the left foot of the patient) of the damaged section of the artery so that the new arterial section grown can, if necessary, be used to take the place of the damaged section of the artery in the event the damaged section is removed.

The first site is on the exterior wall of the artery on one side of the lower end of the damaged section of the artery. A containment system is placed at the first site.

The second site is inside the wall of the artery on the other side of the lower end of the artery.

The genetic carrier solution is heated to a temperature of 98.6 degrees F. 0.25 milliliters of the genetic carrier solution is injected into the containment system at the first site. 0.25 milliliters of the genetic carrier solution is injected at the second site inside the wall of the artery. Care is taken to slowly inject the genetic carrier solution to avoid entry of the solution into the artery such that blood stream will carry away the cDNA in the solution.

After two weeks, an MRI is taken which shows the patient's leg artery. The MRI reveals new growth at the first and second sites.

After four weeks, another MRI is taken which shows the patient's leg artery. The MRI shows that (1) at the first site a new artery is growing adjacent the patient's original leg artery, and (2) at the second site a new section of artery is growing integral with the original artery, i.e., at the second site the new section of artery is lengthening the original artery, much like inserting a new section of hose in a garden hose concentric with the longitudinal axis of the garden hose lengthens the garden hose.

After about eight to twelve weeks, another MRI is taken which shows that the new artery growing adjacent the patient's original artery has grown to a length of about one inch and has integrated itself at each of its ends with the original artery such that blood flows through the new section of artery. The MRI also shows that the new artery at the second site has grown to a length of one-half inch.

In any of the examples of the practice of the invention included herein, cell nutrient culture can be included with the gene, the growth factor, the extracellular matrix, or the environmental factors.

In any of the examples of the practice of the invention included herein, the concept of gene redundancy can be applied. For example, the Examples 1 to 14 concerning a tooth list the genes MSX-1 and MSX-2. These genes differ by only two base pairs. Either gene alone may be sufficient. A further example of redundancy occurs in growth factors. Looking at the Examples 10 to 14, BMP4 or BMP2 alone may be sufficient. Redundancy can also be utilized in connection with transcription factors, extracellular matrices, environmental factors, cell nutrient cultures, physiological nutrient cultures, vectors, promotors, etc.

One embodiment of the invention inserts genetic material (gene, growth factor, ECM, etc.) into the body to induce the formation of an organ. Similar inducing materials inserted ex vivo into or onto a living cell in an appropriate physiological nurturing environment will also induce the growth of an organ. The VCSEL laser allows early detection in a living cell of a morphogenic change indicating that organ formation has been initiated. With properly timed transplantation, organ growth completes itself.

During the ex vivo application of the invention, a gene and/or growth factor is inserted into a cell or a group of cells; an ECM or environmental factor(s) are placed around and in contact with a cell or group of cells; or, genetic material is inserted into a subunit of a cell to induce organ growth. An example of a subunit of a cell is an enucleated cell or a comparable artificially produced environment. In in vivo or ex vivo embodiments of the invention to induce the growth of an organ, the genes, growth factors, or other genetic material, as well as the environmental factors or cells utilized, can come from any desired source.

EXAMPLE 19

Genetically produced materials are inserted in the body to cause the body to grow, reproduce, and replace in vivo a clogged artery in the heart. This is an example of site-specific gene expression. A plasmid expression vector containing an enhancer/promoter is utilized to aid in the transfer of the gene into muscle cells. The enhancer is utilized to drive the specific expression of the transcriptional activator. After the enhancer drives the expression of the transcriptional activator, the transcriptional activator transactivates the muscle/artery genes. Saline is used as a carrier. Cardiac muscle can take up naked DNA injected intramuscularly. Injecting plasmid DNA into cardiac (or skeletal) muscle results in expression of the transgene in cardiac myocytes for several weeks or longer.

Readily available off-the-shelf (RAOTS) cDNA clones for recombinant human VEGF165, isolated from cDNA libraries prepared from HL60 leukemia cells, are assembled in a RAOTS expression plasmid utilizing 736 bp CMV promoter/enhancer to drive VEGF expression. Other RAOTS promoters can be utilized to drive VEGF expression for longer periods of time. Other RAOTS recombinant clones of angiogenic growth factors other than VEGF can be utilized, for example, fibroblast growth factor family, endothelial cell growth factor, etc. Downstream from the VEGF cDNA is an SV40 polyadenylation sequence. These fragments occur in the RAOTS pUC118 vector, which includes an Escherichia coli origin of replication and the Beta lactamase gene for ampicillin resistance.

The RAOTS construct is placed into a RAOTS 3 ml syringe with neutral pH physiologic saline at room temperature (or body temperature of about 37 degrees C). The syringe has a RAOTS 27 gauge needle.

Access to the cardiac muscle is gained by open heart surgery, endoscopic surgery, direction injection of the needle without incision, or by any other desired means. The cardiac muscle immediately adjacent a clogged artery is slowly injected with the RAOTS construct during a five second time period. Injection is slow to avoid leakage through the external covering of muscle cells. About 0.5 ml to 1.0 ml (milliliter) of fluid is injected containing approximately 500 ug phVEGF165 in saline (N=18). The readily available off-the-shelf cDNA clones cause vascular growth which automatically integrates itself with the cardiac muscle. Anatomic evidence of collateral artery formation is observed by the 30th day following injection to the RAOTS construct. One end of the artery integrates itself in the heart wall to receive blood from the heart. The other end of the artery branches into increasing smaller blood vessels to distribute blood into the heart muscle. Once the growth of the new artery is completed, the new artery is left in place in the heart wall. Transplantation of the new artery is not required.

Blood flow through the new artery is calculated in a number of ways. For example, Doppler-derived flow can be determined by electromagnetic flowmeters (using for example, a Doppler Flowmeter sold by Parks Medical Electronic of Aloha, Oregon) both in vitro and in vivo. RAOTS external ultrasound gives a semiquantitative analysis of arterial flow. Also, RAOTS angiograms or any other readily available commercial devices can be utilized.

VEGF gene expression can be evaluated by readily available off-the-shelf polymerase chain reaction (PCR) techniques.

If controls are desired, the plasmid pGSVLacZ containing a nuclear targeted Beta-galactosidase sequence coupled to the simian virus 40 early promoter can be used. To evaluate efficiency, a promoter-matched reporter plasmid, pCMV Beta (available from Clontech of Palo

Alto, California), which encodes Beta-galactosidase under control of CMV promoter/enhancer can be utilized. Other RAOTS products can be utilized if desired.

EXAMPLE 20

A patient, a forty year old African-American female in good health, has been missing tooth number 24 for ten years. The space in her mouth in which her number 24 tooth originally resided is empty. All other teeth except tooth number 24 are present in the patient's mouth. The patient desires a new tooth in the empty "number 24" space in her mouth.

A full thickness mucoperiosteal flap surgery is utilized to expose the bone in the number 24 space. A slight tissue reflection into the number 23 tooth and number 25 tooth areas is carried out to insure adequate working conditions.

A Midwest Quietair handpiece (or other off-the-shelf handpiece) utilizing a #701XXL bur (Dentsply Midwest of Des Plaines, Illinois) (a #700, #557, #558, etc. bur can be utilized if desired) is used to excavate an implant opening or site in the bone. The implant opening is placed midway between the roots of the number 23 and number 25 teeth. The opening ends at a depth which is about fifteen millimeters and which approximates the depth of the apices of the roots of the number 23 and number 25 teeth. Care is taken not to perforate either the buccal or lingual wall of the bone. In addition, care is taken not to perforate or invade the periodontal ligament space of teeth numbers 23 and 25.

An interrupted drilling technique is utilized to avoid overheating the bone when the #701XXL bur is utilized to form the implant opening. During a drilling sequence, the drill is operated in five second increments and the handpiece is permitted to stall. Light pressure and a gentle downward stroke are utilized.

EXAMPLE 36

Example 18 is repeated except that the patient is a 55 year old Caucasian male, and the genetic carrier solution is injected into two sites in the coronary artery of the patient. The first site is on the exterior wall on one side of the artery. The second site is inside the wall of the artery on the other side of the artery. A section of the artery is damaged, is partially blocked, and has a weakened wall. The first and second sites are each below the damaged section of the artery. Similar results are obtained, i.e., a new section of artery grows integral with the original artery, and a new section of artery grows adjacent the original artery. The new section of artery has integrated itself at either end with the original artery so that blood flows through the new section of artery.

THIRD SUPPLEMENTAL DECLARATION

EXHIBIT C

CLAIMS

EXHIBIT C

CLAIMS
APPLICATION SERIAL NO. 09/064,000

Claim 382

A method for producing and integrating tissue consisting of desired soft tissue at a selected site in a body of a human patient comprising:

(a) Placing cells in said body of said human patient;

(b) Forming a bud at said selected site in said body of said human patient; and

(c) Growing said desired soft tissue which integrates itself into said body of said human patient from said bud.

Claim 383

The method of claim 382, wherein said cells are multifactorial and non-specific.

Claim 384

The method of claim 383, wherein said cells comprise stem cells.

Claim 385

The method of claim 382 further comprising forming a new artery.

Claim 386

The method of claim 383 further comprising forming a new artery.

Claim 387

The method of claim 382, wherein said soft tissue comprises mesodermal tissue.

Claim 388	The method of claim 382, wherein said soft tissue comprises an artery.
Claim 389	The method of claim 382, wherein said cells comprise stem cells.
Claim 390	The method of claim 389, wherein said soft tissue comprises an artery.
Claim 391	The method of claim 382, wherein said cells comprise pluripotent cells.
Claim 392	The method of claim 391, wherein said soft tissue comprises an artery.
Claim 393	The method of claim 391, wherein said cells comprise stem cells.
Claim 394	The method of claim 393, wherein said stem cells are multifactorial and non-specific.
Claim 395	The method of claim 382, wherein said cells are injected into said body.
Claim 396	The method of claim 382, wherein said cells are locally placed into said body.
Claim 397	The method of claim 396, wherein said cells comprise stem cells.
Claim 398	The method of claim 396, wherein said cells are injected intramuscularly.
Claim 399	The method of claim 397, wherein said stem cells are injected intramuscularly.

Claim 400	The method of claim 388 further comprising determining blood flow through said new artery.
Claim 401	The method of claim 388 further comprising observing said new artery.
Claim 402	The method of claim 399, wherein said selected site comprises a leg of said patient.
Claim 403	A method for growing and integrating tissue consisting of desired soft tissue at a selected site in a body of a human patient wherein said desired soft tissue comprises a desired artery comprising the steps of:

(a) locally injecting stem cells into said body at said selected site;

(b) forming a bud at said selected site; and

(c) growing said desired artery from said bud wherein said artery integrates itself into said body of said human patient at said selected site. |
| Claim 404 | The method of claim 403, wherein said selected site comprises a damaged site in a leg of said patient and said stem cells are injected intramuscularly. |
| Claim 405 | The method of claim 403, wherein said selected site comprises a damaged site in a heart of said patient and said stem cells are injected intramuscularly. |

Claim 406   The method of claim 402, wherein said desired soft

tissue comprises an artery

EVIDENCE APPENDIX

ITEM NO. 7

Fourth Supplemental Declaration of Dr. Andrew E. Lorincz
cited by Appellant as Exhibit E
in the Response filed November 28, 2007

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Re Application of: James P. Elia	)
	) Group Art Unit: 1646
Serial No.: 09/064,000	)
	) Examiner: Elizabeth C. Kemmerer, Ph.D.
Filed: April 21, 1998	)
	)
For: METHOD FOR GROWTH	)
OF SOFT TISSUE	)
	)

FOURTH SUPPLEMENTAL DECLARATION
OF ANDREW E. LORINCZ, M.D.

I Andrew E. Lorincz declare as follows:

1.   I reside at 16135 NW 243rd Way, High Springs, Florida 32643-3813.

2.   My Curriculum Vitae is attached as Exhibit A to my Declaration of February 12, 2001. Paragraph 4 of my Declaration and my Supplemental Declaration of November 8, 2004 provide additional information regarding my background and experience.

3.   I have read and understood the disclosures of the above-referenced patent application at page 20, line 10 through page 21, line 15; at page 37, lines 19-25; at page 44, line 19 through page 46, line 16; and at page 47, line 22 through page 48, line 15. Such disclosures are the same as I read and understood in my

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previous Declaration. A copy of such disclosures was attached to my Third Supplemental Declaration as Exhibit A.

I have also read and understood additional disclosures of the above-referenced patent application at page 33, lines 8-10; page 40, line 20 through page 43 line 3; page 44, lines 12 and 13; page 48, lines 13-15; page 53, line 1 through page 56, line 25; and page 62, lines 1-10. A copy of such additional disclosures was attached to my Third Supplemental Declaration as Exhibit B.

4. I note that the disclosures referenced in above Paragraph 3 relate to using a growth factor for promoting the growth of soft tissue, and more specifically, to a method of using a cell, such as a stem cell, to grow soft tissue, such as an artery.

5. I have read and understood the claims set forth in the attached Fourth Supplemental Declaration Exhibit A and have been informed that such claims will be concurrently presented in the above-referenced patent application with this Fourth Supplemental Declaration.

6. Based upon above Paragraphs 3-5, it is my opinion that introducing a growth factor, including cells, and more specifically, stem cells, in the body of a human patient will predictably result in the growth of soft tissue, such as an artery, which will integrate itself into pre-existing tissue of the body thereby forming a unified whole.

7.   Based upon above Paragraphs 3-5, it is my opinion that one skilled in the medical arts, armed with the knowledge in such paragraphs, would be able to practice the method set forth in Exhibit A without need for resorting to undue experimentation.

8.   Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 11 - 13 - 07   _Andrew E. Lorincz_
                                              Andrew E. Lorincz, M.D.

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FOURTH SUPPLEMENTAL DECLARATION

EXHIBIT A

CLAIMS

EXHIBIT A

CLAIMS
APPLICATION SERIAL NO. 09/064,000

Claim 403

A method for growing and integrating tissue consisting of desired soft tissue at a selected site in a body of a human patient wherein said desired soft tissue comprises a desired artery comprising the steps of:

(a) locally injecting stem cells into said body at said selected site;

(b) forming a bud at said selected site; and

(c) growing said desired artery from said bud wherein said artery integrates itself into said body of said human patient at said selected site.

Claim 404

The method of claim 403, wherein said selected site comprises a damaged site in a leg of said patient and said stem cells are injected intramuscularly.

Claim 405

The method of claim 403, wherein said selected site comprises a damaged site in a heart of said patient and said stem cells are injected intramuscularly.

Claim 407

The method of claim 403, wherein said stem cell comprises a living stem cell harvested from bone marrow.

Claim 408   The method of claim 407, wherein said bone marrow is from said patient.

Claim 409   The method of claim 403, wherein said stem cell comprises a living stem cell harvested from blood.

Claim 410   The method of claim 409, wherein said blood is from said patient.

Claim 411   The method of claim 403 further comprising determining blood flow through said desired artery.

Claim 412   The method of claim 403 further comprising observing said desired artery.

EVIDENCE APPENDIX

ITEM NO. 8

Declaration of Dr. Richard Heuser filed November 22, 2004

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: James P. Elia)

Serial No.: 09/064,000)

Filed: April 21, 1998)

For: METHOD AND APPARATUS)
        FOR INSTALLATION OF)
        DENTAL IMPLANT)

) Group Art Unit: 1646

) Examiner: Elizabeth Kemmerer, Ph.D.

LETTER

MAIL STOP AF
Commissioner for Patents
P.O. Box 1450
Arlington, VA 22313-1450

Sir:

Enclosed herewith, please find the Declaration of Richard Heuser, M.D.

This Declaration is being submitted in an effort to reduce the number of issues in the instant application and thereby expedite the prosecution thereof.

Respectfully submitted,

Date: November 16, 2004

Gerald K. White

Gerald K. White
Reg. No. 26,611

GERALD K. WHITE & ASSOCIATES, P.C.
205 W. Randolph Street, Suite 835
Chicago, IL 60606
Phone: (312) 920-0588
Fax: (312) 920-0580
Email: gkwpatlaw@aol.com

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Re Application of: James P. Elia)
)
Serial No.: 09/064,000)   Group Art Unit: 1646
)
Filed: April 21, 1998)   Examiner: Elizabeth C. Kemmerer, Ph.D.
)
For: METHOD FOR GROWTH)
    OF SOFT TISSUE)
)

DECLARATION OF RICHARD HEUSER, M.D.

I Richard Heuser declare as follows:

1.   I have offices at 525 North 18th Street, Suite 504, Phoenix, Arizona 85006.

2.   My Curriculum Vitae ("CV") is attached hereto as Exhibit A.

3.   In addition to my CV, I am currently Director of Cardiovascular Research at St. Joseph's Hospital and Medicine Center, and I serve as Clinical Professor of Medicine at University of Arizona College of Medicine. Over the past six years, I have worked in gene therapy, as well as muscle regeneration for the treatment of cardiomyopathy.

In my CV, you will note reference to work that was done with Sulzer Medical involving a rabbit hind limb model to stimulate peripheral vascular disease. I injected a growth mixture that included FGF, etc. into the hind limb model.

In my U.S. Patent No. 6,190,379 entitled "Hot Tip Catheter," I developed a technique to deliver radiofrequency (PMR). In the full embodiment of the patent, I discuss delivery of protein and/or muscle cells in the myocardium using the inventive technique.

I have been involved as a member of the scientific advisory board with the world leader in cardiomyocyte regeneration, Bioheart, Miami Lakes, Florida. This company has been involved with laboratory and clinical trials using skeletal muscle cultured and modified. The sample is then delivered into the myocardium via a surgical or catheter approach.

4.   I have read and understood the disclosures of the above-referenced patent application at page 20, line 10 through page 21, line 15; at page 37, lines 19-25; at page 44, line 19 through page 46, line 16; and at page 47, line 22 through page 48, line 15. A copy of such disclosures is attached hereto as Exhibit B.

5.   I note that the disclosures referenced in above Paragraph 4 relate to using a growth factor for promoting the growth of soft tissue, and more specifically, to a method of using a cell, such as a stem cell, to grow soft tissue, such as an artery.

6.   I am aware of and have considered the definition of *growth factor* in the specification of the above-referenced patent application at page 20, line 10 through page 21, line 15. Such definition is set forth in Exhibit C. Also included in Exhibit C is a definition from the medical dictionary, MEDLINE plus: Merriam-Webster Medical Dictionary, a service of the U.S. NATIONAL LIBRARY OF MEDICINE and the NATIONAL INSTITUTES OF HEALTH. I find that the dictionary definition is consistent with that contained at page 20, line 10 through page 21, line 15 of the above-referenced patent application. I believe that both definitions
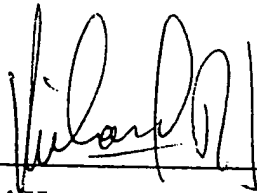
2

are appropriate for use in the field of tissue growth and would be understood by one skilled in the medical arts. Accordingly, I am adopting and utilizing the definition contained in the patent application throughout this declaration.

7.   I have read and understood the claims set forth in Exhibit D and have been informed that such claims are present in the above-referenced patent application. It is my opinion that those skilled in the medical arts, reading such claims would understand that cells including stem cells, are species of living organisms.

8.   The publication in attached Exhibit E illustrates that placement of a growth factor, including cells, and more specifically, stem cells, in a human patient forms soft tissue, such as an artery. This publication reports work performed by reputable, skilled scientists and reputable organizations in the medical arts. Consequently, I believe that these reports would be recognized as clearly valid by one of ordinary skill in the medical arts because they report the results of scientific tests conducted by competent, disinterested third parties with use of proper scientific controls.

9.   Based upon above Paragraphs 4-8, it is my opinion that introducing a growth factor, including cells, and more specifically, stem cells, in the body of a human patient will predictably result in the growth of soft tissue, such as an artery.

10.   Based upon above Paragraphs 4-7, it is my opinion that one skilled in the medical arts, armed with the knowledge in such paragraphs, would be able to practice the method set forth in Exhibit D without need for resorting to undue experimentation.

11.   Declarant states that the above opinion was reached independently.

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Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: _____

_____
Richard Heuser

EXHIBIT A

CURRICULUM VITAE

Curriculum Vitae
Richard Ross Heuser, M.D., F.A.C.C., F.A.C.P.

ADDRESS:
525 North 18th Street, Suite 504
Phoenix, Arizona 85006
(602) 234-0004
(602) 234-0058 (fax)
phoenixheart@earthlink.net

EDUCATION:

1969 - 1972	University of Wisconsin Honors in Chemistry Phi Beta Kappa Evan Helfaer Scholarship in Chemistry
1972 - 1976	University of Wisconsin School of Medicine Graduation with Honors – May 1976 Alpha Omega Alpha Evan Helfaer Scholarship in Medicine

POST GRADUATE TRAINING:

1976 - 1977	Internship in Medicine The Johns Hopkins Hospital Baltimore, Maryland
1977 - 1979	Residency in Medicine The Johns Hopkins Hospital Baltimore, Maryland
1979 - 1981	Fellowship in Cardiology The Johns Hopkins Hospital Baltimore, Maryland

LICENSURE:

State of Arizona, License #19703
State of New Mexico, License #83-220

EMPLOYMENT:

December 2002 - Present	Director of Cardiovascular Research St. Joseph's Hospital and Medical Center Phoenix, Arizona
April 2001 - Present	Cardiac Cath Lab Director St. Luke's Medical Center, Phoenix, Arizona
June 2000 - Present	Medical Director Discovery Alliance, Phoenix, Arizona
1998 - June 2000	Director Phoenix Research Center, Phoenix, Arizona

April 1997 - Present	Medical Director Phoenix Heart Center, Phoenix, Arizona
December 1999 - Present	Director of Research St. Luke's Medical Center, Phoenix, Arizona
April 1997 - December 1999	Director of Research and Education Phoenix Regional Medical Center, Phoenix, Arizona
April 1990 - April 1997	Director of Research and Education Arizona Heart Institute, Phoenix, Arizona
July 1983 - April 1990	Private Practice New Mexico Heart Clinic, Albuquerque, New Mexico
July 1982 - June 1983	Private Practice Houston Cardiovascular Associates, Houston, Texas
June 1981 - July 1982	Instructor in Medicine, Cardiology The Johns Hopkins Hospital, Baltimore, Maryland

PROFESSIONAL APPOINTMENTS:

1981 - July 1982	Instructor in Medicine - Cardiology Division of Cardiology The Johns Hopkins Hospital, Baltimore, Maryland
July 1982 - June 1983	Instructor In Medicine, Cardiology Baylor College of Medicine, Houston, Texas
July 1983 - February 1990	Director, Interventional Cardiology New Mexico Heart Clinic, Albuquerque, New Mexico
April 1984 - June 1986	Clinical Assistant Professor of Medicine University of New Mexico, Albuquerque, New Mexico Director, Medical Residency Program New Mexico Heart Clinic, Albuquerque, New Mexico
June 1986 - April 1990	Clinical Associate Professor of Medicine University of New Mexico, Albuquerque, New Mexico
May 1996 - April 1997	Director, Interventional Cardiology Arizona Heart Institute Foundation, Phoenix, Arizona
Sept 1995 - December 1999	Medical Director - Cardiac Catheterization Laboratory Phoenix Regional Medical Center, Phoenix, Arizona
December 1990 - Present	Clinical Associate Professor of Medicine University of Louisville, Louisville, Kentucky
April 1990 - April 1997	Director of Research and Education Arizona Heart Institute Foundation, Phoenix, Arizona

April 1997 - December 1999   Director of Research and Education
                              Phoenix Regional Medical Center, Phoenix, Arizona

BOARD MEMBERSHIPS:

American Board of Internal Medicine
American Board of Cardiovascular Diseases, Diplomat
American Board of Interventional Cardiovascular Diseases, Diplomat

PROFESSIONAL MEMBERSHIPS:

Fellow, American College of Angiology
Fellow, American College of Cardiology
Fellow, American College of Physicians
Fellow, of the American Heart Association
Fellow, American Society of Cardiovascular Interventions
Fellow, International Society of Cardiovascular Interventions
Fellow, Society for Cardiac Angiography and Interventions
Member, American Association for the Advancement of Science
Member, American Heart Association
Member, American Medical Association
Member, Houston Cardiology Society
Member, Houston Society of Internal Medicine
Member, International Andreas Grüntzig Society
Member, International Network of Interventional Cardiology
Member, International Society for Carotid Artery Therapy
Member, International Society for Minimally Invasive Cardiac Surgery
Member, New Mexico Medical Society
Member, Harris County Medical Society
Member, Texas Medical Association
Member, National Register's Who's Who in Executives and Professionals
Member, Who's Who in Medicine and Healthcare 2002-2003

CLINICAL ADVISORY BOARDS:

Advanced Cardiovascular Systems
USCI
Mansfield Scientific Interventional Board
Medtronic Interventional Vascular
Scientific Advisory Board of International Society of Heart Failure

EDITORIAL BOARDS:

Catheterization and Cardiovascular Diagnosis
Journal of Endovascular Surgery
Cardiovascular Research Foundation/Society or Cardiac Angiography and Interventions
    Abstract Grader TCT

DATA SAFETY BOARDS:

-        ICEM Data Safety Monitoring Board

- Abbott Laboratories Data Safety Monitoring Board for Drug Coated Stent Program, PREFER, A Perspective STUDY to Evaluate the Safety and Efficacy of the ABT-578 coated BiodivYsio® Stent for the Reduction of Resetnosis

CONSULTANT TO:

Editors of the *Annals of Internal Medicine*
Editors of *Catheterization and Cardiovascular Diagnosis*
Editors of *Circulation*
Editors of the *Journal of Invasive Cardiology*
Editors of the *American Journal of Cardiology*
Editors of *Web M.D.*
Annual Scientific Session Program Committee of the American College of Cardiology
Annual Scientific Session Program Committee of the American College of Cardiology
Abstract Advisor for Angioplasty; Stents
Annual International Symposium of Transcatheter Cardiovascular Therapeutics
      Abstract Grader

DEVICE RESEARCH:

Sub-Investigator	ACS Multi-Link Stent Trial Principal Investigator - ACS RX
Principal Investigator	ACT-One Trial Principal Investigator - Angio-Seal Trial
Principal Investigator	Balloon Expandable Intraluminal Stent for Subtotally Occluded Iliac Arteries
Principal Investigator	Bard® Memotherm Carotid Stent Study
Principal Investigator	BARRICADE Trial - The Barrier Approach to Restenosis: Restrict Intima and Curtail Adverse Events (JOMED JOSTENT)
Principal Investigator	BEST Trial
Principal Investigator	BetaCath System Trial
Principal Investigator	Boehringer Ingelheim Pharmaceutics Protocol Comparing Micardis and COZAAR
Principal Investigator	CABERNET Clinical Trial - Carotid Artery Revascularization using the Boston Scientific EPI FiltreWire EX™ and the EndoTex™ NexStent™
Principal Investigator	CADILLAC Trial
Principal Investigator	CAPRICORN Trial
Principal Investigator	CAPTIVE - Cardioshield Application Protects During Transluminal Intervention of Vein Grafts by Reducing Emboli
Principal Investigator	CARDIOMETRICS
Principal Investigator	Carotid Wallstent Trial
Principal Investigator	CAVEAT II Trial
Principal Investigator	Clinical Investigation of the Magnum Wire vs. Standard Guide Wires during Total Occlusion Angioplasty
Principal Investigator	Cook GR II Trial
Principal Investigator	CORDIS Nitinol Carotid Stent And Delivery System for the Treatment of Obstructive Carotid Artery Disease
Principal Investigator	Cordis Carotid Randomized Sapphire
Principal Investigator	Cordis Bilateral AAA Device & Delivery System
Principal Investigator	(CATS) Safe-Steer™ Wire System Coronary Artery Total Occlusion Study
Principal Investigator	CREDO Trial
Principal Investigator	Novoste CUP Trial
Principal Investigator	CVD Accucath Infusion Catheter
Principal Investigator	Duett Closure Devise
Principal Investigator	EndoSonics Cath scanner Oracle - PTCA Catheter

Principal Investigator   EPI FilterWire EX™ System During Transluminal Intervention of
    Saphenous Vein Grafts
Principal Investigator   Extra Stent
Principal Investigator   GREAT - Guided Radio Frequency Energy Ablation of Total Occlusions
    Using the Safe Cross™ Radio Frequency Total Occlusion Crossing System
Principal Investigator   GRIP - Guided Radio Frequency in Peripheral Total Occlusions using the
    Safe-Cross™ Radio Frequency (RF) Total Occlusion (TO) Crossing System
Principal Investigator   HIPS Trial
Principal Investigator   Human Percutaneous Laser Angioplasty of the Coronary Arteries
Principal Investigator   Johnson & Johnson Intracoronary Stent Program Supplement #27
    "New" Delivery System
Principal Investigator   Kensey Nash Hemostatic Puncture Closure Device
Principal Investigator   Mansfield-Boston Scientific Strecker Coronary Stent
Principal Investigator   Medtronic AVE S7 with Discrete Technology Coronary Stent System
Principal Investigator   Medtronic AVE S7 Coronary Stent Registry
Principal Investigator   MOBILE Trial - More Patency with Beta for In-Stent Restenosis in the
    Lower Extremities Trial IDE #G010295; Protocol D00789 Rev B dated 12/01
Principal Investigator   NIR Stent Trial
Principal Investigator   Neurex/Elan Pharmaceuticals Trial
Principal Investigator   PAMI Stent Trial
Principal Investigator   Paragon Stent
Principal Investigator   Paris Radiation Trial
Principal Investigator   PaS Trial
Principal Investigator   Percutaneous Coronary Angioscopy in Unstable Angina
Principal Investigator   Percutaneous Recanalization of Stenotic Human Coronary Arteries
    with Balloon Expandable Intracoronary Stents
Principal Investigator   Percutaneous Recanalization of Stenotic Human Saphenous Vein Bypass
    Graft with Balloon Expandable Intraluminal Stents
Principal Investigator   Percutaneous Thermal Balloon Angioplasty
Principal Investigator   PMR Trial
Principal Investigator   Pravastatin or Atorvastatin Evaluation and Infection Therapy (Prove It)
Principal Investigator   Presto Trial
Principal Investigator   RAVES Trial
Principal Investigator   RESCUE Trial
Principal Investigator   SAFER - Saphenous Vein Graft Angioplasty Free of Emboli Randomized
    Study Using the PercuSurge Guard Wire™ System
Principal Investigator   SAVED Trial
Principal Investigator   Schering-Plough Phase III Study of SCH 58235 in addition to
    Pravastatin compared to placebo in subjects with primary hypercholesterolemia
Principal Investigator   Long-Term, Open-Label, Safety and Tolerability Study of SCH 58235
    in Addition to Pravastatin in Patients with Primary Hypercholesterolemia
Principal Investigator   Schneider WINS Trial
Principal Investigator   SCORES Trial
Principal Investigator   Sepracor Study of Norastemizole in Cardiac Compromised Subjects
Principal Investigator SMART Trial (National PI)
Principal Investigator   SMART: Post-Approval Study
Principal Investigator   SNAPIST - A Phase 2, Safety Study of Systemic Nanoparticle Paclitaxel
    (ABI-007) For In-Stent Restenosis; IND #63,082
Principal Investigator   SOAR - Renal Stent
Principal Investigator   Efficacy and Safety Study of the Oral Direct Thrombin Inhibitor H 376/95
    Compared with Dose-Adjusted Warfarin (Coumadin) In the Prevention of Stroke and Systemic
    Embolic Events in Patients with Atrial Fibrillation (SPORTIF V)
Principal Investigator   STARS Trial
Principal Investigator   START Trial (National PI)
Principal Investigator   STRATUS Trial
Principal Investigator   STRESS III Trial

Principal Investigator	SUMO Trial
Principal Investigator	(SWING) Sound Wave Inhibition of Neointimal Growth
Principal Investigator	Talent Endoluminal Graft (High Risk & Low Risk)
Principal Investigator	Talent Endoluminal Spring Stent-Graft System
Principal Investigator	Tenax-XR Coronary Stent System
Principal Investigator	TITAN Trial
Principal Investigator	Trimedyne Excimer Laser Assisted Percutaneous Coronary Angioplasty
Sub-Investigator	Trimdyne Percutaneous Eclipse Holmium Laser Coronary Angioplasty
Principal Investigator	VeGAS 2 Trial
Principal Investigator	Velocity Trial Principal Investigator - Venus Stent
Co-Investigator	WALLSTENT Study
Principal Investigator	WIKTOR Coronary Stent

PHARMACOLOGY RESEARCH:

Principal Investigator   Abbott rUK Trial

Principal Investigator   Ajinimoto Pharmaceuticals Double-Blind Placebo-Controlled Study of AT-1015 in Patients with Intermittent Claudication due to peripheral arterial disease

Sub-Investigator   Amgen, Inc. Anakinra Trial for Rheumatoid Arthritis

Principal Investigator   Astra Zeneca Pharmaceutical Trial to Evaluate the Safety and Efficacy of XXXX and Atorvastatin

Principal Investigator   Astra Zeneca Trial Open Label Dose Comparison Study to Evaluate the Safety and Efficacy of Rosuvastatin versus Atorvastatin, Pravastatin, and Simvastatin in Subjects with Hypercholesterolemia

Principal Investigator   Parke-Davis and Pfizer Randomized Open-Label Study Comparing the Efficacy of Once Daily Atorvastatin to Simvastatin in Hypercholesterolemic Patients

Principal Investigator   Pilot Study to Evaluate Intracoronary Administration of Activase for the Treatment of Intracoronary Thrombus

Principal Investigator   Artistic Trial

Principal Investigator   AstraZeneca Trial of Niaspan versus New Generation Statin for the Treatment of Type IIB and Type IV Hyperlipidemia

Principal Investigator   AstraZeneca Multicenter Trial for drug (XXX) and Atorvastatin for the Treatment of Hypercholesterolemia

Principal Investigator   BRAVO Trial

Principal Investigator   BioVail Angina & Hypertension Trial

Principal Investigator   CAPRICORN Trial

Principal Investigator   Challenge Trial

Sub-Investigator   Comparison of Lopentol and Omnipaque in Adult Angiocardiography

Sub-Investigator   Comparison of Intravenous Adenosine to Intravenous Placebo in Termination of Spontaneous or Induced Paroxysmal Supraventricular Tachycardia

Principal Investigator   Centocor Chimeric 7E3 Fab

Principal Investigator   COR Therapeutics Randomized Placebo-Controlled Dose Ranging Study of drug (XXXX) in Patients with Atherosclerotic Cardiovascular, Peripheral Vascular, and/or Cerebrovascular Disease

Sub-Investigator   Dose Response Study of Bucindolol in Patients with Congestive Heart Failure

Principal Investigator   Effects of Recombinant Human Superoxide Dismutase in Patients with Acute Myocardial Infarction Subject to Coronary Artery Reperfusion

Sub-Investigator   Eli Lilly - Agitation/Alzheimer's Trial

Principal Investigator   EPILOG Trial

Principal Investigator   ERASER Trial

Principal Investigator   GUSTO Trial

Principal Investigator   A multi-center, randomized, double blind, placebo-and-active controlled Parallel Group Dose-ranging Study of the HMG CoA Reductase Inhibitor, BMS-423526, in the treatment of Hyperlipidemia

Principal Investigator   Study Lovastatin XL with MEVACOR in patients with hypercholesterolemia
Sub-Investigator   Lovastatin Multi-Center Trial
Principal Investigator   Extended Trial of Lovastatin XL for the treatment of hypercholesterolemia
Principal Investigator   Multicenter Double-Blind Placebo controlled trial of drug (XXXX) in
    patients with Type 2 Diabetes and Congestive Heart Failure
Principal Investigator   Effect of LDL-Cholesterol Lowering Beyond Currently Recommended
    Minimum Targets on coronary heart disease (CHD) Recurrence in patients with Pre-Existing
    CHD
Principal Investigator   A Double-Blind, Multi-Center, Randomized, Placebo-Controlled, Parallel
    Group Dosing Study Evaluating the Effects of Nebivolol on Blood Pressure in Patients with Mild
    to moderate Hypertension, NEB 302
Principal Investigator   Parallel Group Extension Study to Determine the Safety and Efficacy of
    Long-Term Nebivolol Exposure in Patients with Mild to Moderate Hypertension NEB 306,
Sub-Investigator   NeoTherapeutics Alzheimer's Disease 2000
Sub-Investigator   NeoTherapeutics Alzheimer's Disease 2001
Principal Investigator   OCTAVE Trial
Sub-Investigator   OCTAVE Trial
Principal Investigator   Pfizer Phase II Multicenter, double-blind placebo controlled randomized
    parallel group dose ranging study of the safety of CP529,414 soft-gel capsules
Principal Investigator   PLAC Trial
Principal Investigator   Protocol 073 Trial
Principal Investigator   Knoll Pharmaceutical Double-Blind Randomized Clinical Trial of Slow
    Release Propafenone (Rythmol-SR®) in the Prevention of Symptomatic Recurrences of Atrial
    Fibrillation
Principal Investigator   PREVAIL - A Phase 2 Multicenter, Double-Blind Placebo-Controlled, Dose-
    Ranging Study to Evaluate the Safety and Efficacy of BO-653 in Prevention of Post-Angioplasty
    Restenosis in Stented Lesions
Principal Investigator   PROVE-IT TIMI 22 - Pravastatin or Atorvastatin Evaluation and Infection
    Therapy
Principal Investigator   PURSUIT Trial
Principal Investigator   QUIET Trial
Principal Investigator   RAFT Trial
Principal Investigator   REPLACE Randomized Evaluation in PCI Linking Angiomax to reduce
    Clinical Events
Sub-Investigator   Safety and Efficacy Study of Burroughs - Wellcome Tissue Plasminogen
    Activator in Patients with Acute Myocardial Infarction
Principal Investigator   A 6-week, open-label, dose-comparison study to evaluate the safety and
    Efficacy of Rosuvastatin versus Atorvastatin, Cerivastatin, pravastatin, and Simvastatin in
    subjects with hypercholesterolemia
Principal Investigator   A 48-week, open-label, non-comparative, Multicentre, Phase IIIb study
    to evaluate the efficacy and safety of the Lipid-Regulating agent Rosuvastatin in the treatment
    of subjects with Fredrickson Type IIa and Type IIb Dyslipidemia, Including Heterozygous
    Familial Hypercholesterolemia
Principal Investigator   SAGE Trial
Sub Investigator   Long Term Open Label Safety and Tolerability Study of SCH58235 inn
    addition to Pravastatin in Patient With Primary Hypercholesterolemia
Principal Investigator   Phase III Double-Blind Efficacy and Safety Study SCH58235 (10 mg) in
    Addition to Pravastatin Compared to Placebo In Subjects with Primary Hypercholesterolemia
Principal Investigator   Phase III Open Label Efficacy and Safety Study SCH58235 (10 mg) in
    Addition to Pravastatin Compared to Placebo in Subjects with Primary Hypercholesterolemia
Principal Investigator   Sepracor Protocol Study of Norastemizole in Cardiac Compromised
    Subjects
Principal Investigator   SPORTIF V - Atrial Fibrillation Trial
Principal Investigator   SWORD Trial
Principal Investigator   Titration-to-Response Trial Comparing Micardis and COZAAR® in
    Patients with Mild-to-moderate Hypertension

Principal Investigator   TNT Trial
Principal Investigator   TREND Trial
Sub-Investigator   VALDECOXIB Trial
Principal Investigator   An Open-Label, Multinational, Multicentre, Extension Trial to Assess the
        Long-Term Safety and Efficacy of ZD4522 in Subjects in the ZD4522 Clinical Trial Program

BASIC RESEARCH:

1990 - 1993   Systematic assessment of Medtronic balloons and guiding catheters in porcine
                 and canine models. Sponsored by Medtronic, Inc.

1990 - 1993   Determination of radiopacity and torquability of Medtronic vascular catheters in
                 porcine models. Sponsored by Medtronic, Inc.

1992 - 1996   Evaluation of Strecker stent in porcine and canine models.
                 Sponsored by Boston Scientific

                 Evaluation of Wiktor stent and stent in porcine and canine models.
                 Sponsored by Medtronic, Inc.

                 Evaluation of NIR stent in porcine models.
                 Sponsored by Cordis Corp.

1990 - 1994   Evaluation of Japan Crescent radiofrequency balloon in porcine model with
                 emphasis on histopathology of heat-produced lesions. Abstract submitted
                 at 1993 AHA Conference.

1993   Evaluation of radiofrequency wire for total coronary occlusions in porcine models:
                 Determining energy limitations. Equipment subsequently licensed to
                 Radius Medical.

1994 - 1997   Training courses for professionals (physicians, engineers, technicians) in techniques
                 and strategies for placement of coronary stents. Five courses sponsored by
                 Johnson & Johnson, Medtronic, Inc. and Cook, Inc.

1997   Efficacy of the Endotex Abdominal Aortic Aneurysm exclusion device in a porcine
                 model gauging ability to exclude renal arteries, ease of placement and
                 radiopacity.   Sponsored by Endotex

1998   Use of percutaneous myocardial revascularization in a porcine model.
                 Sponsored by Cardiogenesis Corporation at Stanford University.

1998 - 1999   Utility of radiofrequency (RF) percutaneous myocardial revascularization
                 in acute and chronic porcine model: Histopathology and angiogenesis
                 related to use of RF alone and in combination with growth factor (VEGF).
                 Results presented at Angiogenesis 1999, Washington, DC.

1999   Development and testing of embolic probe device in porcine model
                 (patent pending). Performed at PRMC and separately at Columbia Presbyterian
                 in New York.

1999   Evaluation of the Medtronic carotid and SVG stent in porcine carotid and
                 saphenous vein graft lesions assessing ease of use and 30-day outcome.
                 Sponsored by Medtronic, Inc.

1999   Development and testing of Protector vascular embolic protection device in

porcine model at Mayo Clinic (device patent pending).

1999   Evaluation of ability of intramuscular growth factor to stimulate angiogenesis in rabbit hindlimb model at 30 and 60 days post-procedure.
Sponsored by Sulzer Medical.

1999   Use of *Vesseal* device to close porcine peripheral artery tears (patent #6,159,197)
Sponsored by Phoenix Heart Center.

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Heuser RR, Maddoux GL, Goss JE, Ramo BW, Raff GL, Shadoff N: Coronary angioplasty for acute mitral regurgitation due to myocardial infarction: A nonsurgical treatment preserving mitral valve integrity. Annals of Internal Medical 1987 Dec;107(6):852-855.

Maddoux GL, Goss JE, Ramo BW, Raff GL, **Heuser RR**, Shadoff N, Wilson JN, Deane WM, Hoyt TW, Fowler BN, Gerety RL, Hoffman AR: Left main coronary artery embolism: A case report. Catheterization and Cardiovascular Diagnosis 1987 Nov-Dec;13(6):394-397.

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Heuser RR: The use of the Holmium: YAG laser in coronary disease: The utility of a unique lensed fiber catheter. The Journal of Interventional Cardiology 1992 Dec;5(4):293-300.

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PATENTS:

1. Method and Apparatus for Treating Body Tissues and Bodily Fluids; Patent granted December 12, 2000 Number: 6,159,197

2. Hot Tip Catheter; Patent granted February 20, 2001 Number: 6,190,379

3. Embolism Prevention Device; Patent granted April 2, 2002 Number: 6,364,900

4. Catheter apparatus and Method for Arterializing a Vein; Patent granted October 15, 2002 Number 6,464,665

5. Methods and apparatus for treating body tissues and bodily fluid vessels; Patent granted October 15, 2002 Number: 6,464,681

6. Catheter for Thermal Evaluation of Arteriosclerotic Plaque; Patent granted March 25, 2003 Number: 6,536,949

7. Small Diameter Snare; Patent granted April 29, 2003 Number: 6,554,842

EXHIBIT
B

DISCLOSURES

APPLICATION
SERIAL NO. 09/064,000

PAGE 20, LINE 10 – PAGE 21, LINE 15

Growth factors can be utilized to induce the growth of "hard tissue" or bone and "soft

tissues" like ectodermal and mesodermal tissues. As used herein, the term growth factor

encompasses compositions and living organisms which promote the growth of hard tissue, such

as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic

matter. The compositions can be genetically produced or manipulated. The living organisms

can be bacteria, viruses, or any other living organism which promote tissue growth. By way of

example and not limitation, growth factors can include platelet-derived growth factor (PDGF),

epidermal growth factor (EGF), fibroblast growth factor (acidic/basis (FGF a,b), interleukins

(IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating

factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF),

bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-

recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate,

be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-

assembles into a nanometer internal diameter pore to build a selective lipid body. Various

enzymes can be utilized for the synthesis of peptides which contain amino acids that control

three-dimensional protein structure and growth. Growth factors can be applied in gels or other

carriers which regulate the rate of release of the growth factors and help maintain the growth

factors and the carrier, at a desired location in the body. Time release capsules, granules, or

other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor an also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which an often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 37, LINES 19-25

Multifactorial and nonspecific cells (such as stem cells and germinal cells) can provide the necessary in vivo and in vitro cascade of genetic material once an implanted master control gene's transcription has been activated. Likewise, any host cell, clone cell, cultured cell, or cell would work. Genetic switches (such as the insect hormone ecdysone) can be used to control genes inserted into humans and animals. These gene switches can also be used in cultured cells or other cells. Gene switches govern whether a gene is on or off making possible precise time of gene activity.

PAGE 44, LINE 19 – PAGE 46, LINE 16

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in connection with any of the implant techniques of the invention, it is understood that a cell nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated (taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which

promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth factors (or other genetic material) are placed. Once the new muscle and blood vessels have

grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

PAGE 47, LINE 22 – PAGE 48, LINE 15

Organs and/or tissues can be formed utilizing the patient's own cells. For example, a skin cell(s) is removed from the intraoral lining of a cheek. The cell is genetically screened to identify DNA damage or other structural and/or functional problems. Any existing prior art genetic screening technique can be utilized. Such methods can utilize lasers, DNA probes, PCR, or any other suitable device. If the cell is damaged, a healthy undamaged cell is, if possible, identified and selected. If a healthy cell can not [sic] be obtained, the damaged cell can be repaired by excision, alkylation, transition or any other desired method. A growth factor(s) is added to the cell to facilitate dedifferentiation and then redifferentiation and morphogenesis into an organ or function specific tissue. Any machine known in the art can be used to check the genetic fitness of the organ and its stage of morphogenesis. A cell nutrient culture may or may not be utilized depending on the desired functional outcome (i.e., growth of an artery, of pancreatic Islet cells, of a heart, etc.) or other circumstances. Replantation can occur at any appropriate stage of morphogenesis. The foregoing can be repeated without the patient's own

cells if universal donor cells such a [sic] germinal cells are utilized. Germinal cells do not require a dedifferentiation. They simply differentiate into desired tissues or organs when properly stimulated. Similarly, the DNA utilized in the foregoing procedure can come from the patient or from any desired source.

During reimplantation one of the patient's own cells is returned to the patient. During implantation, a cell not originally obtained from the patient is inserted on or in the patient.

In the example above, if germinal cells (and in some case, stem cells) are utilized a direct differentiation and morphogenesis into an organ can occur in vivo, ex vivo, or in vitro.

EXHIBIT C

DEFINITIONS

EXHIBIT C

DEFINITIONS

PAGE 20, LINE 10 – PAGE 21, LINE 15

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as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic

matter. The compositions can be genetically produced or manipulated. The living organisms

can be bacteria, viruses, or any other living organism which promote tissue growth. By way of

example and not limitation, growth factors can include platelet-derived growth factor (PDGF),

epidermal growth factor (EGF), fibroblast growth factor (acidic/basis (FGF a,b), interleukins

(IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating

factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF),

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three-dimensional protein structure and growth. Growth factors can be applied in gels or other

carriers which regulate the rate of release of the growth factors and help maintain the growth

factors and the carrier, at a desired location in the body. Time release capsules, granules, or

other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor an also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which an often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

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NATIONAL INSTITUTES OF HEALTH

> **Growth factor:** a substance (as a vitamin B_{12} or an interleukin)
> that promotes growth and especially cellular growth

EXHIBIT D

CLAIMS

EXHIBIT D

CLAIMS
APPLICATION SERIAL NO. 09/064,000

382.   A method for producing a desired soft tissue in a body of a human patient comprising:

    (a)   Placing cells in said body of said human patient;

    (b)   Forming a bud in said body of said human patient; and

    (c)   Growing said desired soft tissue from said bud.

383.   The method of claim 382, wherein said cells are multifactorial and non-specific.

384.   The method of claim 383, wherein said cells comprise stem cells.

385.   The method of claim 382 further comprising forming a new artery.

386.   The method of claim 383 further comprising forming a new artery.

387.   The method of claim 382, wherein said soft tissue comprises mesodermal tissue.

388.   The method of claim 382, wherein said soft tissue comprises an artery.

EXHIBIT E

PUBLICATIONS

Repair of Infarcted Myocardium by Autologous Intracoronary Mononuclear Bone Marrow Cell Transplantation in Humans

Bodo E. Strauer, MD; Michael Brehm, MD; Tobias Zeus, MD; Matthias Köstering, MD; Anna Hernandez, PhD; Rüdiger V. Sorg, PhD; Gesine Kögler, PhD; Peter Wernet, MD

Background—Experimental data suggest that bone marrow–derived cells may contribute to the healing of myocardial infarction (MI). For this reason, we analyzed 10 patients who were treated by intracoronary transplantation of autologous, mononuclear bone marrow cells (BMCs) in addition to standard therapy after MI.

Methods and Results—After standard therapy for acute MI, 10 patients were transplanted with autologous mononuclear BMCs via a balloon catheter placed into the infarct-related artery during balloon dilatation (percutaneous transluminal coronary angioplasty). Another 10 patients with acute MI were treated by standard therapy alone. After 3 months of follow-up, the infarct region (determined by left ventriculography) had decreased significantly within the cell therapy group (from 30 ± 13 to $12\pm7\%$, $P=0.005$) and was also significantly smaller compared with the standard therapy group ($P=0.04$). Likewise, infarction wall movement velocity increased significantly only in the cell therapy group (from 2.0 ± 1.1 to 4.0 ± 2.6 cm/s, $P=0.028$). Further cardiac examinations (dobutamine stress echocardiography, radionuclide ventriculography, and catheterization of the right heart) were performed for the cell therapy group and showed significant improvement in stroke volume index, left ventricular end-systolic volume and contractility (ratio of systolic pressure and end-systolic volume), and myocardial perfusion of the infarct region.

Conclusions—These results demonstrate for the first time that selective intracoronary transplantation of autologous, mononuclear BMCs is safe and seems to be effective under clinical conditions. The marked therapeutic effect may be attributed to BMC-associated myocardial regeneration and neovascularization. (*Circulation.* 2002;106:1913-1918.)

Key Words: myocardial infarction ■ cell transplantation, intracoronary ■ angiogenesis ■ bone marrow ■ myogenesis

Remodeling of the left ventricle after myocardial infarction (MI) represents a major cause of infarct-related heart failure and death. This process depends on acute and chronic transformation of both the necrotic infarct region and the non-necrotic, peri-infarct tissue.[1,2] Despite application of pharmacotherapeutics and mechanical interventions, the cardiomyocytes lost during MI cannot be regenerated. The recent finding that a small population of cardiac muscle cells is able to replicate itself is encouraging but is still consistent with the concept that such regeneration is restricted to viable myocardium.[3]

In animal experiments, attempts to replace the necrotic zone by transplanting other cells (eg, fetal cardiomyocytes or skeletal myoblasts) have invariably succeeded in reconstituting heart muscle structures, ie, myocardium and coronary vessels. However, these cells fail to integrate structurally and do not display characteristic physiological functions.[4-7] Another approach to reverse myocardial remodeling is to repair myocardial tissue by using bone marrow–derived cells. Bone

marrow contains multipotent adult stem cells that show a high capacity for differentiation.[8-10] Experimental studies have shown that bone marrow cells (BMCs) are capable of regenerating infarcted myocardium and inducing myogenesis and angiogenesis; this leads in turn to amelioration of cardiac function in mice and pigs.[11-14] However, procedures based on this phenomenon remain largely uninvestigated in a human clinical setting.

An investigation of one patient receiving autologous skeletal myoblasts into a postinfarction scar during coronary artery bypass grafting revealed improvement of contraction and viability 5 months afterward.[15] Autologous mononuclear BMCs transplanted in a similar surgical setting showed long-term improvement of myocardial perfusion in 3 of 5 patients and no change in 2 patients.[16] However, such studies entail a surgical approach and are therefore associated with well-known perioperative risks. Moreover, this surgical procedure cannot be used with MI. We therefore looked for a nonsurgical, safer mode for transplanting autologous cells

DOI: 10.1161/01.CIR.0000034046.87607.1C

Figure 1. Procedure of cell transplantation into infarcted myocardium in humans. a, The balloon catheter enters the infarct-related artery and is placed above the border zone of the infarction. It is then inflated and the cell suspension is infused at high pressure under stop-flow conditions. b, In this way, cells are transplanted into the infarcted zone via the infarct-related vasculature (red dots). Cells infiltrate the infarcted zone. Blue and white arrows suggest the possible route of migration. c, A supply of blood flow exists within the infarcted zone.[35] The cells are therefore able to reach both the border and the infarcted zone.

into postinfarction tissue. A pilot study from our group demonstrated that intracoronary transplantation of autologous mononuclear BMCs 6 days after MI was associated with a marked decrease in infarct area and an increase in left ventricular (LV) function after 3 and 6 months of follow-up.[17] To confirm these results and validate this promising new therapy for MI, we established a clinical trial involving 20 patients for comparing the safety and bioefficacy of autologous BMC transplantation. All 20 patients underwent standard therapy, and 10 patients received additional intracoronary cell transplantation. All 20 patients were followed up for 3 months.

Methods

Patient Population

All 20 patients had suffered transmural infarction according to World Health Organization criteria with the involvement of the left anterior descending coronary artery (n=4), left circumflex coronary artery (n=3), or right coronary artery (n=13). Mean duration of infarct pain was 12±10 hours before invasive diagnostics and therapy. Patients had to be <70 years old and were excluded if one of the following criteria were met: screening >72 hours after infarction, cardiac shock, severe comorbidity, alcohol or drug dependency, or excessive travel distance to the study center.

After right and left heart catheterization, coronary angiography, and left ventriculography, mechanical treatment was initiated with recanalization of the infarct-related artery by balloon angioplasty (n=20) and subsequent stent implantation (n=19). All patients were monitored in our intensive care unit, and no arrhythmogenic events or hemodynamic impairments were recorded in either patient group.

All 20 patients were briefed in detail about the procedure of BMC transplantation. Informed consent was obtained from 10 patients, who formed the cell therapy group, whereas 10 patients who refused additional cell therapy served as controls. The local ethics committee of the Heinrich-Heine-University, Düsseldorf, approved the study protocol. All procedures conformed to institutional guidelines.

Before taking part in rehabilitation programs, all patients left the hospital with standard medication consisting of acetylsalicylic acid, an ACE inhibitor, a β-blocker, and a statin.

Bone Marrow Aspiration, Isolation, and Cultivation

Seven (±2) days after acute coronary angiography, bone marrow (~40 mL) was aspirated under local anesthesia from ilium of cell therapy patients (n=10). Mononuclear BMCs were isolated by Ficoll density separation on Lymphocyte Separation Medium (BioWhittaker) before the erythrocytes were lysed with H_2O. For overnight

cultivation, 1×10⁶ BMCs/mL were placed in Teflon bags (Vuelife, Cell Genix) and cultivated in X-Vivo 15 Medium (BioWhittaker) supplemented with 2% heat-inactivated autologous plasma. The next day, BMCs were harvested and washed 3 times with heparinized saline before final resuspension in heparinized saline. Viability was 93±3%. Heparinization and filtration (cell strainer, FALCON) was carried out to prevent cell clotting and microembolization during intracoronary transplantation. The mean number of mononuclear cells harvested after overnight culture was 2.8×10⁷; this consisted of 0.65±0.4% AC133-positive cells and 2.1±0.28% CD34-positive cells. All microbiological tests of the clinically used cell preparations proved negative. As a viability and quality ex vivo control, 1×10⁵ cells grown in H5100 medium (Stem Cell Technology) were found to be able to generate mesenchymal cells in culture.

Intracoronary Transplantation of BMCs

Five to nine days after onset of acute infarction, cells were directly transplanted into the infarcted zone (Figure 1). This was accomplished with the use of a balloon catheter, which was placed within the infarct-related artery. After exact positioning of the balloon at the site of the former infarct-vessel occlusion, percutaneous transluminal coronary angioplasty (PTCA) was performed 6 to 7 times for 2 to 4 minutes each. During this time, intracoronary cell transplantation via the balloon catheter was performed, using 6 to 7 fractional high-pressure infusions of 2 to 3 mL cell suspension, each of which contained 1.5 to 4×10⁶ mononuclear cells. PTCA thoroughly prevented the backflow of cells and at the same time produced a stop-flow beyond the site of the balloon inflation to facilitate high-pressure infusion of cells into the infarcted zone. Thus, prolonged contact time for cellular migration was allowed.[18]

Functional Assessment of Hemodynamics

After 3 months, all 20 patients were followed up by left heart catheterization, left ventriculography, and coronary angiography. Ejection fraction, infarct region, and regional wall movement of the infarcted zone during ejection were determined by left ventriculography. Ejection fraction was measured with Quantcor software (Siemens). To quantify infarction wall movement velocity, 5 axes were placed perpendicular to the long axis in the main akinetic or dyskinetic segment of the ventricular wall. Relative systolic and diastolic lengths were measured, and the mean difference was divided by the systolic duration (in seconds). To quantify the infarct region, the centerline method according to Sheehan was used.[19] All hemodynamic investigations were obtained by two independent observers.

In the cell therapy group before and 3 months after cell transplantation, additional examinations for measuring hemodynamics and myocardial perfusion included dobutamine stress echocardiography, radionuclide ventriculography, catheterization of the right heart, and

TABLE 1. Baseline Characteristics of the Patients

Clinical Data	Cell Therapy	Standard Therapy	P
Characteristics			
No. of patients	10	10	...
Age, y	49±10	50±6	NS
Sex	Male	Male	...
Onset of infarction before angioplasty, h	10±8	13±11	NS
Coronary angiography			
No. of diseased vessels	1.7±0.9	2.1±0.7	NS
No. of patients with LAD/LCX/RCA as the affected vessel	4/1/5	0/2/8	...
No. of patients with stent implantation	9	10	...
Laboratory parameters			
Creatinine kinase, U/L	1138±1170	1308±1187	NS
Creatinine kinase-MB, U/L	106±72	124±92	NS
Bone marrow puncture after angioplasty, d	7±2	...	...
Mononuclear bone marrow cells, n (×10⁷)	2.8±2.2	...	...

Values are mean±SD or number of patients.
NS indicates not significant; LAD, left anterior descending coronary artery; LCX, left circumflex coronary artery; and RCA, right coronary artery.

stress-redistribution-reinjection ^{201}thallium scintigraphy. The contractility index P_{sys}/ESV was calculated by dividing LV systolic pressure (P_{sys}) by end-systolic volume (ESV). Perfusion defect was calculated by scintigraphic bull's-eye technique. Each examination was performed according to standard protocols.

There were no complications or side effects determined in any patient throughout the diagnostic or therapeutic procedure or within the 3-month follow-up period.

Statistical Analysis

All data are presented as mean±SD. Statistical significance was accepted when P was <0.05. Discrete variables were compared as rates, and comparisons were made by χ^2 analysis. Intra-individual comparison of baseline versus follow-up continuous variables was performed with a paired t test. Comparison of nonparametric data between the two groups was performed with Wilcoxon test and Mann-Whitney test. Statistical analysis was performed with SPSS for Windows (version 10.1).

Results

Clinical data between the two groups did not differ significantly. The range of creatinine kinase levels was slightly but not significantly higher in the standard therapy group than it was in the cell therapy group (Table 1).

Comparison of the 2 groups 3 months after cell or standard therapy showed several significant differences in LV dynamics, according to the global and regional analysis of left ventriculogram. The infarct region as a percentage of hypo-kinetic, akinetic, or dyskinetic segments of the circumference of the left ventricle decreased significantly in the cell therapy group (from 30±13 to 12±7%, P=0.005). It was also significantly smaller compared with the standard therapy group after 3 months (P=0.04). Within the standard therapy group, only a statistically nonsignificant decrease from 25±8 to 20±11% could be seen. Wall movement velocity over the infarct region rose significantly in the cell therapy group (from 2.0±1.1 to 4.0±2.6 cm/s, P=0.028) but not in the standard therapy group (from 1.8±1.3 to 2.3±1.6 cm/s, P=NS). No significant difference was observed between the

two groups. Ejection fraction increased in both groups, albeit nonsignificantly (from 57±8 to 62±10% in the cell therapy group and from 60±7 to 64±7% in the standard therapy group) (Table 2).

Further significant improvement could also be seen on additional analysis of the cell therapy group alone. Perfusion defect was considerably decreased by 26% in the cell therapy group (from 174±99 to 128±71 cm², P=0.016, assessed by ^{201}thallium scintigraphy) (Figure 2). Parallel to the reduction in perfusion defect, improvement (Table 3) could also be seen in:

(1) Cardiac function, as revealed by increase in stroke volume index (from 49±7 to 56±7 mL/m², P=0.010) and ejection fraction (from 51±14 to 53±13%, P=NS).

(2) Cardiac geometry, as shown by decreases in both end-diastolic (from 158±20 to 143±30 mL, P=NS) and end-systolic volume (from 82±26 to 67±21 mL, P=0.011). Radionuclide ventriculography was used to acquire the data.

(3) Contractility as evaluated by an increase in the velocity of circumferential fiber shortening (from 20.5±4.2 to 24.4±7.7 mm/s, P=NS, assessed by stress echocardiography) and by a marked increase in the ratio of systolic pressure to end-systolic volume (from 1.81±1.44 to 2.27±1.72 mm Hg/mL, P=0.005).

Discussion

The present report describes the first clinical trial of intracoronary, autologous, mononuclear BMC transplantation for improving heart function and myocardial perfusion in patients after acute MI. The results demonstrate that transplanted autologous BMCs may lead to repair of infarcted tissue when applied during the immediate postinfarction period. These results also show that the intracoronary approach of BMC transplantation seems to represent a novel

TABLE 2. Comparison of Cell Therapy and Standard Therapy Groups

	Cell Therapy	Standard Therapy	P
No. of patients	10	10	...
Infarct region as functional defect			
Hypokinetic, akinetic, or dyskinetic region at 0 mo, %	30±13	25±8	NS
Hypokinetic, akinetic, or dyskinetic region at 3 mo, %	12±7	20±11	0.04
P	0.005	NS	...
Contractility Indices			
Infarction wall movement velocity at 0 mo, cm/s	2.0±1.1	1.8±1.3	NS
Infarction wall movement velocity at 3 mo, cm/s	4.0±2.6	2.3±1.6	NS
P	0.028	NS	...
Hemodynamic data			
LV ejection fraction at 0 mo, %	57±8	60±7	NS
LV ejection fraction at 3 mo, %	62±10	64±7	NS
P	NS	NS	...

NS indicates not significant; 0 mo, zero months, which means the time of infarction; 3 mo, 3 months, which means the time of the follow-up examinations. All data were obtained according to analysis of left ventriculogram.

and effective therapeutic procedure for concentrating and/or depositing infused cells within the region of interest.

Neogenesis of both cardiomyocytes and coronary capillaries with some functional improvement has been shown recently by several investigators using bone marrow–derived cells in experimental infarction.[11–14,18,20–23] Moreover, trans-endothelial migration from the coronary capillaries and in-corporation of cells into heart muscle has been observed experimentally.[3,12,24–26] Until now, clinical data only existed for the cell therapy of surgically treated chronic ischemic heart disease.[15,16] Our aim was to transform the encouraging results from animal models to a safe clinical setting. The most crucial questions we had to address while designing and

Figure 2. Improved myocardial perfusion of infarcted anterior wall 3 months after intracoronary cell transplantation subsequent to an acute anterior wall infarction detected by [201]thallium scintigraphy. The images on the left (A, D, sagittal) and in the middle (B, E) show the long axis, whereas those on the right (C, F, frontal) show the short axis of the heart. Initially the anterior wall, with green-colored apical and anterior regions, had reduced myocardial perfusion (A, B, C). Three months after cell transplantation the same anterior wall, now yellow in color, revealed a significant improvement in myocardial perfusion (D, E, F). All illustrations depict the exercise phase.

realizing this trial were: (1) What cell population should we deliver? (2) Which application method is the most efficient? (3) When should the cells be transplanted?

In recent years, several laboratories have shown that environmentally dictated changes of fate (transdetermination) are not restricted to stem cells but may also involve progenitor cells at different steps of a given differentiation pathway (transdifferentiation). Moreover, mesenchymal stem cells may represent an ideal cell source for treating different diseases.[27] Adult, mononuclear BMCs contain such stem and progenitor cells (≤1%), eg, mesodermal progenitor cells, hematopoietic progenitor cells, and endothelial progenitor cells. In several animal infarction models it has been shown that: (1) Bone marrow hemangioblasts contribute to the formation of new vessels; (2) bone marrow hematopoietic stem cells differentiate into cardiomyocytes, endothelium,

TABLE 3. Cardiac Function Analysis at 3-Month Follow-Up

	Before Cell Therapy	3 Months After Cell Therapy	P
No. of patients	10	10	...
Hemodynamic data			
LV ejection fraction, %	51±14	53±13	NS
Stroke volume index, mL/m²	49±7	56±7	0.010
Cardiac geometry			
LV end-diastolic volume, mL	158±20	143±30	NS
LV end-systolic volume, mL	82±26	67±21	0.011
Contractility indices			
Circumferential fiber shortening, mm/s	20.5±4.2	24.4±7.7	NS
P_{sys}/ESV, mm Hg/mL	1.81±1.44	2.27±1.72	0.005
Infarct region as perfusion defect			
[201]Thallium scintigraphy, cm²	174±99	128±71	0.016

NS indicates not significant.

and smooth muscle cells[8-13]; (3) BMCs give rise to mesodermal progenitor cells that differentiate to endothelial cells[28]; and (4) endothelial progenitors can transdifferentiate into beating cardiomyocytes.[29] Thus, several different fractions of mononuclear BMCs may contribute to the regeneration of necrotic myocardium and vessels. In order to utilize this large and perhaps heterogeneous regenerative potential, we decided to use all mononuclear cells from the bone marrow aspirate as a whole, rather than a subpopulation. No further expansion was performed because experimental data have revealed a dramatic decline in the homing capacity of in vitro amplified hematopoietic stem or progenitor cells.[30]

The second question was how to deliver the cells most efficiently. When given intravenously, only a very small fraction of infused cells can reach the infarct region after the following injection: assuming a normal coronary blood flow of 80 mL/min per 100 g of LV weight, a quantity of 160 mL per left ventricle (assuming a regular LV mass of ~200 g) will flow per minute.[31,32] This corresponds to only about 3% of cardiac output (assuming a cardiac output of 5000 mL/min).[31] Therefore, intravenous application would require many circulation passages to enable infused cells to come into contact with the infarct-related artery. Throughout this long circulation and recirculation time, homing of cells to other organs could considerably reduce the numbers of cells dedicated to cell repair in the infarcted zone. Thus, supplying the entire complement of cells by intracoronary administration obviously seems to be advantageous for the tissue repair of infarcted heart muscle and may also be superior to intraventricular injection,[33] because all cells are able to flow through the infarcted and peri-infarcted tissue during the immediate first passage. Accordingly, by this intracoronary procedure the infarct tissue and the peri-infarct zone can be enriched with the maximum available amount of cells at all times.

As stem cells differentiate into more mature types of progenitor cells, it is thought that a special microenvironment in so-called niches regulates cell activity by providing specific combinations of cytokines and by establishing direct cellular contact. For successful long-term engraftment, at least some stem cells have to reach their niches, a process referred to as homing. Mouse experiments have shown that significant numbers of BMCs appear in liver, spleen, and bone marrow after intravenous injection.[34] To offer the BMCs the best chance of finding their niche within the myocardium, a selective intracoronary delivery route was chosen. Presumably, therefore, fewer cells were lost by extraction toward organs of secondary interest by this first pass-like effect. To facilitate transendothelial passage and migration into the infarcted zone, cells were infused by high-pressure injection directly into the necrotic area, and the balloon was kept inflated for 2 to 3 minutes; the cells were not washed away immediately under these conditions.

The time point for delivery was chosen as 7 to 8 days after infarction onset for the following reasons:

(1) In dogs, infarcted territory becomes rich in capillaries and contains enlarged, pericyte-poor "mother vessels" and endothelial bridges 7 days after myocardial ischemia and reperfusion. Twenty-eight days later, a significant muscular vessel wall has already formed.[35] Thus, with such timing, cells may be able to reach the worst

damaged parts and at the same time salvage tissue. Transendothelial cell migration may also be enhanced because an adequate muscular coat is not yet formed.

(2) Until now, only one animal study has attempted to determine the optimum time for cardiomyocyte transplantation to maximize myocardial function after LV injury. Adult rat hearts were cryoinjured and fetal rat cardiomyocytes were transplanted immediately, 2 weeks later, and 4 weeks later. The authors discussed the inflammatory process, which is strongest in the first days after infarction, as being responsible for the negative results after immediate cell transplantation, and they assumed that the best results seen after 2 weeks may have been due to transplantation before scar expansion.[36] Until now, however, no systematic experiments have been performed with BMCs to correlate the results of transplantation with the length of such a time delay.

(3) Another important variable is the inflammatory response in MI, which seems to be a superbly orchestrated interaction of cells, cytokines, growth factors and extracellular matrix proteins mediating myocardial repair. In the first 48 hours, debridement and formation of a fibrin-based provisional matrix predominates before a healing phase ensues.[37-40] Moreover, vascular endothelial growth factor is at its peak concentration 7 days after MI, and the decline of adhesion molecules (intercellular adhesion molecules, vascular cell adhesion molecules) does not take place before days 3 to 4 after MI. We assumed that transplantation of mononuclear BMCs within the "hot" phase of post-MI inflammation might lead them to take part in the inflammation cascade rather than the formation of functional myocardium and vessels.

Taking all of this into account, we can conclude that cell transplantation within the first 5 days after acute infarction is not possible for logistical reasons and is not advisable because of the inflammatory process. On the other hand, transplantation 2 weeks after infarction scar formation seems to reduce the benefit of cell transplantation. Although the ideal time point for transplantation remains to be defined, it is most likely between days 7 and 14 after the onset of MI, as in the present study.

This trial was designed as a phase I safety and feasibility trial, meaning that no control group is necessarily required. However, to validate the results, we correlated them with those obtained from 10 patients who refused to get additional cell therapy and thus received standard therapy alone. We are aware of the fact that such a comparison does not reach the power of a randomly allocated, blinded control group. However, the significant improvement with regard to infarct region, hemodynamics (stroke volume index), cardiac geometry (LV end-systolic volume), and contractility (P_{sys}/ESV and infarction wall movement velocity) did confirm a positive effect of the additional cell therapy because the changes observed in the standard therapy group failed to reach significance.

Another important factor for interpreting the results is time interval between onset of symptoms and revascularization of the infarct-related artery by angioplasty; this represents a crucial determinant of LV recovery. For patients with acute MI, it has

been shown that if the time interval is >4 hours, no significant changes in ejection fraction, regional wall motion, or ESV are observed after 6-month follow-up by echocardiography and angiography.[41] None of our 20 patients was treated by angioplasty within 4 hours after onset of symptoms. Our average time interval was 12±10 hours. Thus, PTCA-induced improvement of LV function can be nearly excluded; indeed, the only mild and nonsignificant changes within the standard therapy group are consistent with the above-mentioned data.[41] In contrast, the cell therapy group showed considerable and significant improvement in the same parameters, which may be attributed to BMC-mediated coronary angioneogenesis and cardiomyoneogenesis.

These results show that transplantation of autologous BMCs, as well as the intracoronary approach, represent a novel and effective therapeutic procedure for the repair of infarcted myocardium. For this method of therapy, no ethical problems exist, and no side effects were observed at any point of time. The therapeutic benefit for the patient's heart seems to prevail. However, further experimental studies, controlled prospective clinical trials, and variations of cell preparations are required to define the role of this new approach for the therapy of acute MI in humans.

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EVIDENCE APPENDIX

ITEM NO. 9

Supplemental Declaration of Dr. Heuser filed June 20, 2005

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Re Application of: James P. Elia   )

Serial No.: 09/064,000   ) Group Art Unit: 1646

Filed: April 21, 1998   ) Examiner: Elizabeth C. Kemmerer, Ph.D.

For: METHOD FOR GROWTH
     OF SOFT TISSUE

SUPPLEMENTAL DECLARATION OF RICHARD HEUSER, M.D.

I Richard Heuser declare as follows:

1.   I have offices at 500 West Thomas Road, Suite 900, Phoenix, Arizona 85013.

2.   This Supplemental Declaration is submitted in addition to my previous Declaration, dated November 11, 2004. No changes are made to my previous Declaration.

3.   My Curriculum Vitae is attached as Exhibit A to my Declaration of November 11, 2004.

4.   It is my understanding that the Examiner in charge of the above-identified patent application is also in the Examiner in charge of co-pending patent application Serial No. 09/794,456. In an Advisory Action dated November 26, 2004, for aforesaid Serial No. 09/794456, the Examiner questioned my qualification to render my opinions in my previous Declarations filed in such application. It is my further understanding that the Examiner reviewed my U.S. Patent No.

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6,190,379 and did not find mention of delivery of any substance to the myocardium nor the word "cell." Also, the Examiner questioned my role in the cell delivery portion of Bioheart's laboratory and clinical trials using skeletal muscle cultured and modified. For the instant application, I provide the following information to respond to the Examiner's questions.

5.   Regarding, U.S. Patent No. 6,190,379, the following is stated in Paragraph 3 of my Declaration:

> In my U.S. Patent No. 6,190,379 entitled "Hot Tip Catheter," I developed a technique to deliver radiofrequency (PMR). In the full embodiment of the patent, I discuss delivery of protein and/or muscle cells in the myocardium using the inventive technique.

By the above statement, I meant that the device shown in the patent has been used for the delivery of protein and/or muscle cells to the myocardium. At a presentation at the Angiogenesis Meeting in 1999 in Washington, D.C., we described this use of growth factors in a pig model with the development of neo vascularization. Moreover, I have had discussions with Bioheart regarding the use of my U.S. Patent No. 6,190,379 for delivery of cells.

Regarding my work at Bioheart, the following is stated in my Declaration:

> I have been involved as a member of the scientific advisory board with the world leader in cardiomyocyte regeneration, Bioheart, Miami Lakes, Florida. This company has been involved with laboratory and clinical trials using skeletal muscle cultured and modified. The sample is then delivered into the myocardium via a surgical or catheter approach.

To provide further information regarding the Examiner's questioning my involvement with Bioheart, I am a Scientific Advisory Board Member and in such role advise Bioheart throughout its pre-clinical and clinical work involving the
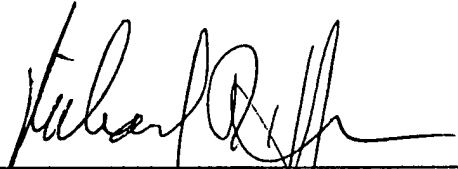
delivery of skeletal muscle cells into the myocardium. I am also an investigator with Bioheart's Phase 3 clinical trials in the United States. Such trials have not yet commenced.

6.   Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: _____

_____
Richard Heuser, M.D., F.A.C.C., F.A.C.P.

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EVIDENCE APPENDIX

ITEM NO. 10

Second Supplemental Declaration of Dr. Heuser cited by Appellant as Exhibit C in the Response filed June 26, 2006

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Re Application of: James P. Elia)
)   Group Art Unit: 1646
Serial No.: 09/064,000)
)   Examiner: Elizabeth C. Kemmerer
Filed: April 21, 1998)
)
For: METHOD FOR GROWTH)
     OF SOFT TISSUE)

SECOND SUPPLEMENTAL DECLARATION
OF RICHARD HEUSER, M.D., F.A.C.C., F.A.C.P.

I Richard Heuser declare as follows:

1. I have offices at 525 North 18th Street, Suite 504, Phoenix, Arizona 85006.

2. My Curriculum Vitae is attached as Exhibit A to my Declaration of November 11, 2004. Paragraph 3 of my Declaration and my Supplemental Declaration of February 15, 2005 provide additional information regarding my background and experience.

3. I have read and understood the disclosures of the above-referenced patent application at page 20, line 10 through page 21, line 15; at page 37, lines 19-25; at page 44, line 19 through page 46, line 16; and at page 47, line 22 through page 48, line 15. Such disclosures are the same as I read and understood in my previous Declaration. A copy of such disclosures is attached hereto as Second Supplemental Declaration Exhibit A.

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I have also read and understood additional disclosures of the above-referenced patent application at page 33, lines 8-10; page 40, line 20 through page 43 line 3; page 44, lines 12 and 13; page 48, lines 13-15; page 53, line 1 through page 56, line 25; and page 62, lines 1-10. A copy of such additional disclosures is attached hereto as Second Supplemental Declaration Exhibit B.

4.   I note that the disclosures referenced in above Paragraph 3 relate to using a growth factor for promoting the growth of soft tissue, and more specifically, to a method of using a cell, such as a stem cell, to grow soft tissue, such as an artery.

5.   I have read and understood the claims set forth in the attached Second Supplemental Declaration Exhibit C and have been informed that such claims will be concurrently presented in the above-referenced patent application with this Second Supplemental Declaration.

6.   Based upon above Paragraphs 3-5, it is my opinion that introducing a growth factor, including cells, and more specifically, stem cells, in the body of a human patient will predictably result in the growth of soft tissue, such as an artery, which will integrate itself into pre-existing tissue of the body thereby forming a unified whole.

7.   Based upon above Paragraphs 3-5, it is my opinion that one skilled in the medical arts, armed with the knowledge in such paragraphs, would be able to practice the method set forth in Exhibit C without need for resorting to undue experimentation.

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8.   Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both   (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: ___6||9|06___   _____

Richard Heuser

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SECOND SUPPLEMENTAL DECLARATION

EXHIBIT A

DISCLOSURES

EXHIBIT A

DISCLOSURES
APPLICATION SERIAL NO. 09/064,000

PAGE 20, LINE 10 – PAGE 21, LINE 15

Growth factors can be utilized to induce the growth of "hard tissue" or bone and "soft

tissues" like ectodermal and mesodermal tissues. As used herein, the term growth factor

encompasses compositions and living organisms which promote the growth of hard tissue, such

as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic

matter. The compositions can be genetically produced or manipulated. The living organisms

can be bacteria, viruses, or any other living organism which promote tissue growth. By way of

example and not limitation, growth factors can include platelet-derived growth factor (PDGF),

epidermal growth factor (EGF), fibroblast growth factor (acidic/basis (FGF a,b), interleukins

(IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating

factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF),

bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-

recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate,

be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-

assembles into a nanometer internal diameter pore to build a selective lipid body. Various

enzymes can be utilized for the synthesis of peptides which contain amino acids that control

three-dimensional protein structure and growth. Growth factors can be applied in gels or other

carriers which regulate the rate of release of the growth factors and help maintain the growth

factors and the carrier, at a desired location in the body. Time release capsules, granules, or

other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor an also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which an often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 37, LINES 19-25

Multifactorial and nonspecific cells (such as stem cells and germinal cells) can provide the necessary in vivo and in vitro cascade of genetic material once an implanted master control gene's transcription has been activated. Likewise, any host cell, clone cell, cultured cell, or cell would work. Genetic switches (such as the insect hormone ecdysone) can be used to control genes inserted into humans and animals. These gene switches can also be used in cultured cells or other cells. Gene switches govern whether a gene is on or off making possible precise time of gene activity.

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in connection with any of the implant techniques of the invention, it is understood that a cell nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated (taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which

promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth factors (or other genetic material) are placed. Once the new muscle and blood vessels have

grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

PAGE 47, LINE 22 – PAGE 48, LINE 15

Organs and/or tissues can be formed utilizing the patient's own cells. For example, a skin cell(s) is removed from the intraoral lining of a cheek. The cell is genetically screened to identify DNA damage or other structural and/or functional problems. Any existing prior art genetic screening technique can be utilized. Such methods can utilize lasers, DNA probes, PCR, or any other suitable device. If the cell is damaged, a healthy undamaged cell is, if possible, identified and selected. If a healthy cell can not [sic] be obtained, the damaged cell can be repaired by excision, alkylation, transition or any other desired method. A growth factor(s) is added to the cell to facilitate dedifferentiation and then redifferentiation and morphogenesis into an organ or function specific tissue. Any machine known in the art can be used to check the genetic fitness of the organ and its stage of morphogenesis. A cell nutrient culture may or may not be utilized depending on the desired functional outcome (i.e., growth of an artery, of pancreatic Islet cells, of a heart, etc.) or other circumstances. Replantation can occur at any appropriate stage of morphogenesis. The foregoing can be repeated without the patient's own

cells if universal donor cells such a [sic] germinal cells are utilized. Germinal cells do not require a dedifferentiation. They simply differentiate into desired tissues or organs when properly stimulated. Similarly, the DNA utilized in the foregoing procedure can come from the patient or from any desired source.

During reimplantation one of the patient's own cells is returned to the patient. During implantation, a cell not originally obtained from the patient is inserted on or in the patient.

In the example above, if germinal cells (and in some case, stem cells) are utilized a direct differentiation and morphogenesis into an organ can occur in vivo, ex vivo, or in vitro.

SECOND SUPPLEMENTAL DECLARATION

EXHIBIT B

DISCLOSURES

EXHIBIT B

DISCLOSURES
APPLICATION SERIAL NO. 09/064,000

PAGE 33, LINES 8-10

Morphogenesis or morphogenetics is the origin and evolution of morphological characters and is the growth and differentiation of cells and tissues during development.

PAGE 40, LINE 20 – PAGE 43, LINE 3

EXAMPLE 11

MSX-1 and MSX-2 are the homeobox genes that control the generation and growth of a tooth. A sample of skin tissue is removed from the patient and the MSX-1 and MXS-2 homeobox gene(s) are removed from skin tissue cells. The genes are stored in an appropriate nutrient culture medium.

BMP-2 and BMP-4 growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

MXS-1 and MXS-2 transcription factors are obtained which will initiate the expression of the MXS-1 and MXS-2 homeobox genes.

The MXS-1 and MXS-2 transcription factors, BMP-2 and BMP-4 bone morphogenic proteins, and MXS-1 and MXS-2 genes are added to the nutrient culture medium along with the living stem cells.

EXAMPLE 12

Example 11 is repeated except that the transcription factors bind to a receptor complex in the stem cell nucleus.

EXAMPLE 13

Example 11 is repeated except that the MXS-1 and MXS-2 transcription factors are not utilized. The transcription of the MXS-1 and MXS-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 14

Example 13 is repeated except that the stem cells are starved and the transcription of the MXS-1 and MXS-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 15

WT-1 and PAX genes are obtained from a sample of skin tissue is removed from the patient. The genes are stored in an appropriate nutrient culture medium. PAX genes produce PAX-2 and other transcription factors.

BMP-7 and other kidney related BMP growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees.

- B2 -

The temperature of the culture medium can be varied as desired but ordinarily is between 40 and 102 degrees F.

The WT-1 and PAX genes, and BMP-7 and other kidney BMPS are added to the nutrient culture medium along with the living stem cells.

A primitive kidney germ is produced. The kidney germ is transplanted in the patient's body near a large artery. As the kidney grows, its blood supply will be derived from the artery.

EXAMPLE 16

The Aniridia gene is obtained from a sample of skin tissue is removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

The Aniridia transcription factor and growth factors and the Aniridia gene are added to the nutrient culture medium along with the living stem cells.

A primitive eye germ is produced. The kidney germ is transplanted in the patient's body near the optic nerve. As the kidney grows, its blood supply will be derived from nearby arteries.

EXAMPLE 17

The Aniridia gene is obtained from a sample of skin tissue is removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained and added to the nutrient culture medium.

An eye germ develops. A branch of the nearby maxillary artery is translocated to a position adjacent the eye germ to promote the development of the eye germ. The eye germ matures into an eye which receives its blood supply from the maxillary artery.

The term "cell nutrient culture" as used herein can include any or any combination of the following: the extracellular matrix; conventional cell culture nutrients; and/or, a cell nutrient such as a vitamin. As such, the cell nutrient culture can be two-dimensional, three dimensional, or simply a nutrient, and is useful in promoting the processes of cellular dedifferentiation, redifferentiation, differentiation, growth, and development.

PAGE 44, LINES 12– 13

An organ, as used herein, consists of two or more kinds of tissues joined into one structure that has a certain task.

PAGE 48, LINES 13– 15

In the example above, if germinal cells (and in some cases, stem cells) are utilized a direct differentiation and morphogenesis into an organ can occur in vivo, ex vivo, or in vitro.

EXAMPLE 18

A 36 year old Caucasian male experiences pain in his left leg. A medical examination reveals a damaged one inch long section of a large artery in his left leg. The examination also reveals that this damaged section of the artery is nearly completely clogged with plaque and that the wall of the artery is weakened. The weakening in the arterial wall makes attempting to clean out the artery risky and also makes it risky to attempt to insert a stent in the artery.

Recombinant cDNA encoded to combine with a cell ribosome to produce the human growth factor VEGF is assembled into a eukaryotic expression plasmid. The recombinant cDNA is from cDNA libraries prepared from HL60 leukemia cells and is known to cause the growth of arteries. The plasmid is maintained at a room temperature of 76 degrees F.

The clones are placed in 1.0 milliliters of a normal saline carrier solution at a room temperature of 76 degrees F to produce an genetic carrier solution. The genetic carrier solution contains about 250 ug of the cDNA clones. A nutrient culture can, if desired, be utilized in conjunction with or in place of the saline carrier. Each clone is identical. If desired, only a single clone can be inserted in the normal saline carrier solution. The saline carrier solution comprises 0.09% by weight sodium chloride in water. A saline carrier solution is selected because it will not harm the DNA clone.

Two sites are selected for injection of the genetic carrier solution. While the selection of sites can vary as desired, the sites are selected at the lower end (the end nearest the left foot of the patient) of the damaged section of the artery so that the new arterial section grown can, if necessary, be used to take the place of the damaged section of the artery in the event the damaged section is removed.

The first site is on the exterior wall of the artery on one side of the lower end of the damaged section of the artery. A containment system is placed at the first site.

The second site is inside the wall of the artery on the other side of the lower end of the artery.

The genetic carrier solution is heated to a temperature of 98.6 degrees F. 0.25 milliliters of the genetic carrier solution is injected into the containment system at the first site. 0.25 milliliters of the genetic carrier solution is injected at the second site inside the wall of the artery. Care is taken to slowly inject the genetic carrier solution to avoid entry of the solution into the artery such that blood stream will carry away the cDNA in the solution.

After two weeks, an MRI is taken which shows the patient's leg artery. The MRI reveals new growth at the first and second sites.

After four weeks, another MRI is taken which shows the patient's leg artery. The MRI shows that (1) at the first site a new artery is growing adjacent the patient's original leg artery, and (2) at the second site a new section of artery is growing integral with the original artery, i.e., at the second site the new section of artery is lengthening the original artery, much like inserting a new section of hose in a garden hose concentric with the longitudinal axis of the garden hose lengthens the garden hose.

After about eight to twelve weeks, another MRI is taken which shows that the new artery growing adjacent the patient's original artery has grown to a length of about one inch and has integrated itself at each of its ends with the original artery such that blood flows through the new section of artery. The MRI also shows that the new artery at the second site has grown to a length of one-half inch.

In any of the examples of the practice of the invention included herein, cell nutrient culture can be included with the gene, the growth factor, the extracellular matrix, or the environmental factors.

In any of the examples of the practice of the invention included herein, the concept of gene redundancy can be applied. For example, the Examples 1 to 14 concerning a tooth list the genes MSX-1 and MSX-2. These genes differ by only two base pairs. Either gene alone may be sufficient. A further example of redundancy occurs in growth factors. Looking at the Examples 10 to 14, BMP4 or BMP2 alone may be sufficient. Redundancy can also be utilized in connection with transcription factors, extracellular matrices, environmental factors, cell nutrient cultures, physiological nutrient cultures, vectors, promotors, etc.

One embodiment of the invention inserts genetic material (gene, growth factor, ECM, etc.) into the body to induce the formation of an organ. Similar inducing materials inserted ex vivo into or onto a living cell in an appropriate physiological nurturing environment will also induce the growth of an organ. The VCSEL laser allows early detection in a living cell of a morphogenic change indicating that organ formation has been initiated. With properly timed transplantation, organ growth completes itself.

During the ex vivo application of the invention, a gene and/or growth factor is inserted into a cell or a group of cells; an ECM or environmental factor(s) are placed around and in contact with a cell or group of cells; or, genetic material is inserted into a subunit of a cell to induce organ growth. An example of a subunit of a cell is an enucleated cell or a comparable artificially produced environment. In in vivo or ex vivo embodiments of the invention to induce the growth of an organ, the genes, growth factors, or other genetic material, as well as the environmental factors or cells utilized, can come from any desired source.

EXAMPLE 19

Genetically produced materials are inserted in the body to cause the body to grow, reproduce, and replace in vivo a clogged artery in the heart. This is an example of site-specific gene expression. A plasmid expression vector containing an enhancer/promoter is utilized to aid in the transfer of the gene into muscle cells. The enhancer is utilized to drive the specific expression of the transcriptional activator. After the enhancer drives the expression of the transcriptional activator, the transcriptional activator transactivates the muscle/artery genes. Saline is used as a carrier. Cardiac muscle can take up naked DNA injected intramuscularly. Injecting plasmid DNA into cardiac (or skeletal) muscle results in expression of the transgene in cardiac myocytes for several weeks or longer.

Readily available off-the-shelf (RAOTS) cDNA clones for recombinant human VEGF165, isolated from cDNA libraries prepared from HL60 leukemia cells, are assembled in a RAOTS expression plasmid utilizing 736 bp CMV promoter/enhancer to drive VEGF expression. Other RAOTS promoters can be utilized to drive VEGF expression for longer periods of time. Other RAOTS recombinant clones of angiogenic growth factors other than VEGF can be utilized, for example, fibroblast growth factor family, endothelial cell growth factor, etc. Downstream from the VEGF cDNA is an SV40 polyadenylation sequence. These fragments occur in the RAOTS pUC118 vector, which includes an Escherichia coli origin of replication and the Beta lactamase gene for ampicillin resistance.

The RAOTS construct is placed into a RAOTS 3 ml syringe with neutral pH physiologic saline at room temperature (or body temperature of about 37 degrees C). The syringe has a RAOTS 27 gauge needle.

Access to the cardiac muscle is gained by open heart surgery, endoscopic surgery, direction injection of the needle without incision, or by any other desired means. The cardiac muscle immediately adjacent a clogged artery is slowly injected with the RAOTS construct during a five second time period. Injection is slow to avoid leakage through the external covering of muscle cells. About 0.5 ml to 1.0 ml (milliliter) of fluid is injected containing approximately 500 ug phVEGF165 in saline (N=18). The readily available off-the-shelf cDNA clones cause vascular growth which automatically integrates itself with the cardiac muscle. Anatomic evidence of collateral artery formation is observed by the 30th day following injection to the RAOTS construct. One end of the artery integrates itself in the heart wall to receive blood from the heart. The other end of the artery branches into increasing smaller blood vessels to distribute blood into the heart muscle. Once the growth of the new artery is completed, the new artery is left in place in the heart wall. Transplantation of the new artery is not required.

Blood flow through the new artery is calculated in a number of ways. For example, Doppler-derived flow can be determined by electromagnetic flowmeters (using for example, a Doppler Flowmeter sold by Parks Medical Electronic of Aloha, Oregon) both in vitro and in vivo. RAOTS external ultrasound gives a semiquantitative analysis of arterial flow. Also, RAOTS angiograms or any other readily available commercial devices can be utilized.

VEGF gene expression can be evaluated by readily available off-the-shelf polymerase chain reaction (PCR) techniques.

If controls are desired, the plasmid pGSVLacZ containing a nuclear targeted Beta-galactosidase sequence coupled to the simian virus 40 early promoter can be used. To evaluate efficiency, a promoter-matched reporter plasmid, pCMV Beta (available from Clontech of Palo

Alto, California), which encodes Beta-galactosidase under control of CMV promoter/enhancer can be utilized. Other RAOTS products can be utilized if desired.

EXAMPLE 20

A patient, a forty year old African-American female in good health, has been missing tooth number 24 for ten years. The space in her mouth in which her number 24 tooth originally resided is empty. All other teeth except tooth number 24 are present in the patient's mouth. The patient desires a new tooth in the empty "number 24" space in her mouth.

A full thickness mucoperiosteal flap surgery is utilized to expose the bone in the number 24 space. A slight tissue reflection into the number 23 tooth and number 25 tooth areas is carried out to insure adequate working conditions.

A Midwest Quietair handpiece (or other off-the-shelf handpiece) utilizing a #701XXL bur (Dentsply Midwest of Des Plaines, Illinois) (a #700, #557, #558, etc. bur can be utilized if desired) is used to excavate an implant opening or site in the bone. The implant opening is placed midway between the roots of the number 23 and number 25 teeth. The opening ends at a depth which is about fifteen millimeters and which approximates the depth of the apices of the roots of the number 23 and number 25 teeth. Care is taken not to perforate either the buccal or lingual wall of the bone. In addition, care is taken not to perforate or invade the periodontal ligament space of teeth numbers 23 and 25.

An interrupted drilling technique is utilized to avoid overheating the bone when the #701XXL bur is utilized to form the implant opening. During a drilling sequence, the drill is operated in five second increments and the handpiece is permitted to stall. Light pressure and a gentle downward stroke are utilized.

EXAMPLE 36

Example 18 is repeated except that the patient is a 55 year old Caucasian male, and the genetic carrier solution is injected into two sites in the coronary artery of the patient. The first site is on the exterior wall on one side of the artery. The second site is inside the wall of the artery on the other side of the artery. A section of the artery is damaged, is partially blocked, and has a weakened wall. The first and second sites are each below the damaged section of the artery. Similar results are obtained, i.e., a new section of artery grows integral with the original artery, and a new section of artery grows adjacent the original artery. The new section of artery has integrated itself at either end with the original artery so that blood flows through the new section of artery.

SECOND SUPPLEMENTAL DECLARATION

EXHIBIT C

CLAIMS

EXHIBIT C

CLAIMS
APPLICATION SERIAL NO. 09/064,000

Claim 382

A method for producing and integrating tissue consisting of a desired soft tissue at a selected site in a body of a human patient comprising:

(a) Placing cells in said body of said human patient;

(b) Forming a bud at said selected site in said body of said human patient; and

(c) Growing said desired soft tissue which integrates itself into said body of said human patient from said bud.

Claim 383

The method of claim 382, wherein said cells are multifactorial and non-specific.

Claim 384

The method of claim 383, wherein said cells comprise stem cells.

Claim 385

The method of claim 382 further comprising forming a new artery.

Claim 386

The method of claim 383 further comprising forming a new artery.

Claim 387

The method of claim 382, wherein said soft tissue comprises mesodermal tissue.

Claim 388	The method of claim 382, wherein said soft tissue comprises an artery.
Claim 389	The method of claim 382, wherein said cells comprise stem cells.
Claim 390	The method of claim 389, wherein said soft tissue comprises an artery.
Claim 391	The method of claim 382, wherein said cells comprise pluripotent cells.
Claim 392	The method of claim 391, wherein said soft tissue comprises an artery.
Claim 393	The method of claim 391, wherein said cells comprise stem cells.
Claim 394	The method of claim 393, wherein said stem cells are multifactorial and non-specific.
Claim 395	The method of claim 382, wherein said cells are injected into said body.
Claim 396	The method of claim 382, wherein said cells are locally placed into said body.
Claim 397	The method of claim 396, wherein said cells comprise stem cells.
Claim 398	The method of claim 396, wherein said cells are injected intramuscularly.
Claim 399	The method of claim 397, wherein said stem cells are injected intramuscularly.

Claim 400   The method of claim 388 further comprising determining

                 blood flow through said new artery.

Claim 401   The method of claim 388 further comprising observing

                 said new artery.

Claim 402   The method of claim 399, wherein said selected site

                 comprises a leg of said patient.

EVIDENCE APPENDIX

ITEM NO. 11

Third Supplemental Declaration of Dr. Heuser cited by
Appellant as Exhibit C in the Response filed April 30, 2007

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Re Application of: James P. Elia)
) Group Art Unit: 1646
Serial No.: 09/064,000   )
) Examiner: Elizabeth C. Kemmerer
Filed: April 21, 1998   )
)
For: METHOD FOR GROWTH)
  OF SOFT TISSUE   )

THIRD SUPPLEMENTAL DECLARATION
OF RICHARD HEUSER, M.D., F.A.C.C., F.A.C.P.

I Richard Heuser declare as follows:

1. I have offices at 500 West Thomas Road, Suite 900, Phoenix, Arizona 85013.

2. My Curriculum Vitae was attached as Exhibit A to my Declaration of November 11, 2004. Paragraph 3 of my Declaration and my Supplemental Declaration of February 15, 2005 provide additional information regarding my background and experience.

3. I have read and understood the disclosures of the above-referenced patent application at page 20, line 10 through page 21, line 15; at page 37, lines 19-25; at page 44, line 19 through page 46, line 16; and at page 47, line 22 through page 48, line 15. A copy of such disclosures is attached hereto as Third Supplemental Declaration Exhibit A.

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I have also read and understood additional disclosures of the above-referenced patent application at page 33, lines 8-10; page 40, line 20 through page 43 line 3; page 44, lines 12 and 13; page 48, lines 13-15; page 49, lines 18-22; page 53, line 1 through page 56, line 25; and page 62, lines 1-10;. A copy of such additional disclosures is attached hereto as Third Supplemental Declaration Exhibit B.

4.   I note that the disclosures referenced in above Paragraph 3 relate to using a growth factor for promoting the growth of soft tissue, and more specifically, to a method of using a cell, such as a stem cell, to grow soft tissue, such as an artery.

5.   I have read and understood the claims set forth in the attached Third Supplemental Declaration Exhibit C and have been informed that such claims will be concurrently presented in the above-referenced patent application with this Third Supplemental Declaration.

6.   Based upon above Paragraphs 3-5, it is my opinion that introducing a growth factor, including cells, and more specifically, stem cells, in the body of a human patient will predictably result in the growth of soft tissue, such as an artery, which will integrate itself into pre-existing tissue of the body thereby forming a unified whole.

7.   Based upon above Paragraphs 3-5, it is my opinion that one skilled in the medical arts, armed with the knowledge in such paragraphs, would be able to practice the method set forth in Exhibit C without need for resorting to undue experimentation.

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8.   Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: _____

_____
Richard Heuser

3

THIRD SUPPLEMENTAL
DECLARATION

EXHIBIT A

DISCLOSURES

EXHIBIT A

DISCLOSURES
APPLICATION SERIAL NO. 09/064,000

PAGE 20, LINE 10 – PAGE 21, LINE 15

Growth factors can be utilized to induce the growth of "hard tissue" or bone and "soft tissues" like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basis (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

-A1-

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor an also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which an often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 37, LINES 19-25

Multifactorial and nonspecific cells (such as stem cells and germinal cells) can provide the necessary in vivo and in vitro cascade of genetic material once an implanted master control gene's transcription has been activated. Likewise, any host cell, clone cell, cultured cell, or cell would work. Genetic switches (such as the insect hormone ecdysone) can be used to control genes inserted into humans and animals. These gene switches can also be used in cultured cells or other cells. Gene switches govern whether a gene is on or off making possible precise time of gene activity.

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in connection with any of the implant techniques of the invention, it is understood that a cell nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated (taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which

promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth factors (or other genetic material) are placed. Once the new muscle and blood vessels have

grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

PAGE 47, LINE 22 – PAGE 48, LINE 15

Organs and/or tissues can be formed utilizing the patient's own cells. For example, a skin cell(s) is removed from the intraoral lining of a cheek. The cell is genetically screened to identify DNA damage or other structural and/or functional problems. Any existing prior art genetic screening technique can be utilized. Such methods can utilize lasers, DNA probes, PCR, or any other suitable device. If the cell is damaged, a healthy undamaged cell is, if possible, identified and selected. If a healthy cell can not [sic] be obtained, the damaged cell can be repaired by excision, alkylation, transition or any other desired method. A growth factor(s) is added to the cell to facilitate dedifferentiation and then redifferentiation and morphogenesis into an organ or function specific tissue. Any machine known in the art can be used to check the genetic fitness of the organ and its stage of morphogenesis. A cell nutrient culture may or may not be utilized depending on the desired functional outcome (i.e., growth of an artery, of pancreatic Islet cells, of a heart, etc.) or other circumstances. Replantation can occur at any appropriate stage of morphogenesis. The foregoing can be repeated without the patient's own

cells if universal donor cells such a [sic] germinal cells are utilized. Germinal cells do not require a dedifferentiation. They simply differentiate into desired tissues or organs when properly stimulated. Similarly, the DNA utilized in the foregoing procedure can come from the patient or from any desired source.

During reimplantation one of the patient's own cells is returned to the patient. During implantation, a cell not originally obtained from the patient is inserted on or in the patient.

In the example above, if germinal cells (and in some case, stem cells) are utilized a direct differentiation and morphogenesis into an organ can occur in vivo, ex vivo, or in vitro.

THIRD SUPPLEMENTAL DECLARATION

EXHIBIT B

ADDITIONAL DISCLOSURES

DISCLOSURES
APPLICATION SERIAL NO. 09/064,000

PAGE 33, LINES 8-10

Morphogenesis or morphogenetics is the origin and evolution of morphological characters and is the growth and differentiation of cells and tissues during development.

PAGE 40, LINE 20 – PAGE 43, LINE 3

EXAMPLE 11

MSX-1 and MSX-2 are the homeobox genes that control the generation and growth of a tooth. A sample of skin tissue is removed from the patient and the MSX-1 and MXS-2 homeobox gene(s) are removed from skin tissue cells. The genes are stored in an appropriate nutrient culture medium.

BMP-2 and BMP-4 growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

MXS-1 and MXS-2 transcription factors are obtained which will initiate the expression of the MXS-1 and MXS-2 homeobox genes.

The MXS-1 and MXS-2 transcription factors, BMP-2 and BMP-4 bone morphogenic proteins, and MXS-1 and MXS-2 genes are added to the nutrient culture medium along with the living stem cells.

EXAMPLE 12

Example 11 is repeated except that the transcription factors bind to a receptor complex in the stem cell nucleus.

EXAMPLE 13

Example 11 is repeated except that the MXS-1 and MXS-2 transcription factors are not utilized. The transcription of the MXS-1 and MXS-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 14

Example 13 is repeated except that the stem cells are starved and the transcription of the MXS-1 and MXS-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 15

WT-1 and PAX genes are obtained from a sample of skin tissue is removed from the patient. The genes are stored in an appropriate nutrient culture medium. PAX genes produce PAX-2 and other transcription factors.

BMP-7 and other kidney related BMP growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees.

The temperature of the culture medium can be varied as desired but ordinarily is between 40 and 102 degrees F.

The WT-1 and PAX genes, and BMP-7 and other kidney BMPS are added to the nutrient culture medium along with the living stem cells.

A primitive kidney germ is produced. The kidney germ is transplanted in the patient's body near a large artery. As the kidney grows, its blood supply will be derived from the artery.

EXAMPLE 16

The Aniridia gene is obtained from a sample of skin tissue is removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

The Aniridia transcription factor and growth factors and the Aniridia gene are added to the nutrient culture medium along with the living stem cells.

A primitive eye germ is produced. The kidney germ is transplanted in the patient's body near the optic nerve. As the kidney grows, its blood supply will be derived from nearby arteries.

EXAMPLE 17

The Aniridia gene is obtained from a sample of skin tissue is removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained and added to the nutrient culture medium.

An eye germ develops. A branch of the nearby maxillary artery is translocated to a position adjacent the eye germ to promote the development of the eye germ. The eye germ matures into an eye which receives its blood supply from the maxillary artery.

The term "cell nutrient culture" as used herein can include any or any combination of the following: the extracellular matrix; conventional cell culture nutrients; and/or, a cell nutrient such as a vitamin. As such, the cell nutrient culture can be two-dimensional, three dimensional, or simply a nutrient, and is useful in promoting the processes of cellular dedifferentiation, redifferentiation, differentiation, growth, and development.

PAGE 44, LINES 12– 13

An organ, as used herein, consists of two or more kinds of tissues joined into one structure that has a certain task.

PAGE 48, LINES 13– 15

In the example above, if germinal cells (and in some cases, stem cells) are utilized a direct differentiation and morphogenesis into an organ can occur in vivo, ex vivo, or in vitro.

PAGE 49, LINES 18-22

Avascular necrosis can be corrected with the insertion of a gene(s) and/or growth factor or other genetic material in the body. For example, avascular necrosis is diagnosed near a joint space. VEGF or BMP genes, or VEFG or BMP growth factors produced by VEFG or BMP genes, respectively, or any other desired genetic based material can be inserted to regrow blood vessels and/or bone.

PAGE 53, LINE 1 – PAGE 56, LINE 25

EXAMPLE 18

A 36 year old Caucasian male experiences pain in his left leg. A medical examination reveals a damaged one inch long section of a large artery in his left leg. The examination also reveals that this damaged section of the artery is nearly completely clogged with plaque and that the wall of the artery is weakened. The weakening in the arterial wall makes attempting to clean out the artery risky and also makes it risky to attempt to insert a stent in the artery.

Recombinant cDNA encoded to combine with a cell ribosome to produce the human growth factor VEGF is assembled into a eukaryotic expression plasmid. The recombinant cDNA is from cDNA libraries prepared from HL60 leukemia cells and is known to cause the growth of arteries. The plasmid is maintained at a room temperature of 76 degrees F.

The clones are placed in 1.0 milliliters of a normal saline carrier solution at a room temperature of 76 degrees F to produce an genetic carrier solution. The genetic carrier solution contains about 250 ug of the cDNA clones. A nutrient culture can, if desired, be utilized in conjunction with or in place of the saline carrier. Each clone is identical. If desired, only a single clone can be inserted in the normal saline carrier solution. The saline carrier solution

comprises 0.09% by weight sodium chloride in water. A saline carrier solution is selected because it will not harm the DNA clone.

Two sites are selected for injection of the genetic carrier solution. While the selection of sites can vary as desired, the sites are selected at the lower end (the end nearest the left foot of the patient) of the damaged section of the artery so that the new arterial section grown can, if necessary, be used to take the place of the damaged section of the artery in the event the damaged section is removed.

The first site is on the exterior wall of the artery on one side of the lower end of the damaged section of the artery. A containment system is placed at the first site.

The second site is inside the wall of the artery on the other side of the lower end of the artery.

The genetic carrier solution is heated to a temperature of 98.6 degrees F. 0.25 milliliters of the genetic carrier solution is injected into the containment system at the first site. 0.25 milliliters of the genetic carrier solution is injected at the second site inside the wall of the artery. Care is taken to slowly inject the genetic carrier solution to avoid entry of the solution into the artery such that blood stream will carry away the cDNA in the solution.

After two weeks, an MRI is taken which shows the patient's leg artery. The MRI reveals new growth at the first and second sites.

After four weeks, another MRI is taken which shows the patient's leg artery. The MRI shows that (1) at the first site a new artery is growing adjacent the patient's original leg artery, and (2) at the second site a new section of artery is growing integral with the original artery, i.e., at the second site the new section of artery is lengthening the original artery, much like inserting

a new section of hose in a garden hose concentric with the longitudinal axis of the garden hose lengthens the garden hose.

After about eight to twelve weeks, another MRI is taken which shows that the new artery growing adjacent the patient's original artery has grown to a length of about one inch and has integrated itself at each of its ends with the original artery such that blood flows through the new section of artery. The MRI also shows that the new artery at the second site has grown to a length of one-half inch.

In any of the examples of the practice of the invention included herein, cell nutrient culture can be included with the gene, the growth factor, the extracellular matrix, or the environmental factors.

In any of the examples of the practice of the invention included herein, the concept of gene redundancy can be applied. For example, the Examples 1 to 14 concerning a tooth list the genes MSX-1 and MSX-2. These genes differ by only two base pairs. Either gene alone may be sufficient. A further example of redundancy occurs in growth factors. Looking at the Examples 10 to 14, BMP4 or BMP2 alone may be sufficient. Redundancy can also be utilized in connection with transcription factors, extracellular matrices, environmental factors, cell nutrient cultures, physiological nutrient cultures, vectors, promotors, etc.

One embodiment of the invention inserts genetic material (gene, growth factor, ECM, etc.) into the body to induce the formation of an organ. Similar inducing materials inserted ex vivo into or onto a living cell in an appropriate physiological nurturing environment will also induce the growth of an organ. The VCSEL laser allows early detection in a living cell of a morphogenic change indicating that organ formation has been initiated. With properly timed transplantation, organ growth completes itself.

During the ex vivo application of the invention, a gene and/or growth factor is inserted into a cell or a group of cells; an ECM or environmental factor(s) are placed around and in contact with a cell or group of cells; or, genetic material is inserted into a subunit of a cell to induce organ growth. An example of a subunit of a cell is an enucleated cell or a comparable artificially produced environment. In in vivo or ex vivo embodiments of the invention to induce the growth of an organ, the genes, growth factors, or other genetic material, as well as the environmental factors or cells utilized, can come from any desired source.

EXAMPLE 19

Genetically produced materials are inserted in the body to cause the body to grow, reproduce, and replace in vivo a clogged artery in the heart. This is an example of site-specific gene expression. A plasmid expression vector containing an enhancer/promoter is utilized to aid in the transfer of the gene into muscle cells. The enhancer is utilized to drive the specific expression of the transcriptional activator. After the enhancer drives the expression of the transcriptional activator, the transcriptional activator transactivates the muscle/artery genes. Saline is used as a carrier. Cardiac muscle can take up naked DNA injected intramuscularly. Injecting plasmid DNA into cardiac (or skeletal) muscle results in expression of the transgene in cardiac myocytes for several weeks or longer.

Readily available off-the-shelf (RAOTS) cDNA clones for recombinant human VEGF165, isolated from cDNA libraries prepared from HL60 leukemia cells, are assembled in a RAOTS expression plasmid utilizing 736 bp CMV promoter/enhancer to drive VEGF expression. Other RAOTS promoters can be utilized to drive VEGF expression for longer periods of time. Other RAOTS recombinant clones of angiogenic growth factors other than VEGF can be utilized, for example, fibroblast growth factor family, endothelial cell growth

factor, etc. Downstream from the VEGF cDNA is an SV40 polyadenylation sequence. These fragments occur in the RAOTS pUC118 vector, which includes an Escherichia coli origin of replication and the Beta lactamase gene for ampicillin resistance.

The RAOTS construct is placed into a RAOTS 3 ml syringe with neutral pH physiologic saline at room temperature (or body temperature of about 37 degrees C). The syringe has a RAOTS 27 gauge needle.

Access to the cardiac muscle is gained by open heart surgery, endoscopic surgery, direction injection of the needle without incision, or by any other desired means. The cardiac muscle immediately adjacent a clogged artery is slowly injected with the RAOTS construct during a five second time period. Injection is slow to avoid leakage through the external covering of muscle cells. About 0.5 ml to 1.0 ml (milliliter) of fluid is injected containing approximately 500 ug phVEGF165 in saline (N=18). The readily available off-the-shelf cDNA clones cause vascular growth which automatically integrates itself with the cardiac muscle. Anatomic evidence of collateral artery formation is observed by the 30th day following injection to the RAOTS construct. One end of the artery integrates itself in the heart wall to receive blood from the heart. The other end of the artery branches into increasing smaller blood vessels to distribute blood into the heart muscle. Once the growth of the new artery is completed, the new artery is left in place in the heart wall. Transplantation of the new artery is not required.

Blood flow through the new artery is calculated in a number of ways. For example, Doppler-derived flow can be determined by electromagnetic flowmeters (using for example, a Doppler Flowmeter sold by Parks Medical Electronic of Aloha, Oregon) both in vitro and in vivo. RAOTS external ultrasound gives a semiquantitative analysis of arterial flow. Also, RAOTS angiograms or any other readily available commercial devices can be utilized.

VEGF gene expression can be evaluated by readily available off-the-shelf polymerase chain reaction (PCR) techniques.

If controls are desired, the plasmid pGSVLacZ containing a nuclear targeted Beta-galactosidase sequence coupled to the simian virus 40 early promoter can be used. To evaluate efficiency, a promoter-matched reporter plasmid, pCMV Beta (available from Clontech of Palo Alto, California), which encodes Beta-galactosidase under control of CMV promoter/enhancer can be utilized. Other RAOTS products can be utilized if desired.

EXAMPLE 20

A patient, a forty year old African-American female in good health, has been missing tooth number 24 for ten years. The space in her mouth in which her number 24 tooth originally resided is empty. All other teeth except tooth number 24 are present in the patient's mouth. The patient desires a new tooth in the empty "number 24" space in her mouth.

A full thickness mucoperiosteal flap surgery is utilized to expose the bone in the number 24 space. A slight tissue reflection into the number 23 tooth and number 25 tooth areas is carried out to insure adequate working conditions.

A Midwest Quietair handpiece (or other off-the-shelf handpiece) utilizing a #701XXL bur (Dentsply Midwest of Des Plaines, Illinois) (a #700, #557, #558, etc. bur can be utilized if desired) is used to excavate an implant opening or site in the bone. The implant opening is placed midway between the roots of the number 23 and number 25 teeth. The opening ends at a depth which is about fifteen millimeters and which approximates the depth of the apices of the roots of the number 23 and number 25 teeth. Care is taken not to perforate either the buccal or lingual wall of the bone. In addition, care is taken not to perforate or invade the periodontal ligament space of teeth numbers 23 and 25.

An interrupted drilling technique is utilized to avoid overheating the bone when the #701XXL bur is utilized to form the implant opening. During a drilling sequence, the drill is operated in five second increments and the handpiece is permitted to stall. Light pressure and a gentle downward stroke are utilized.

PAGE 62, LINES 1–10

EXAMPLE 36

Example 18 is repeated except that the patient is a 55 year old Caucasian male, and the genetic carrier solution is injected into two sites in the coronary artery of the patient. The first site is on the exterior wall on one side of the artery. The second site is inside the wall of the artery on the other side of the artery. A section of the artery is damaged, is partially blocked, and has a weakened wall. The first and second sites are each below the damaged section of the artery. Similar results are obtained, i.e., a new section of artery grows integral with the original artery, and a new section of artery grows adjacent the original artery. The new section of artery has integrated itself at either end with the original artery so that blood flows through the new section of artery.

THIRD SUPPLEMENTAL DECLARATION

EXHIBIT C

CLAIMS

EXHIBIT C

CLAIMS
APPLICATION SERIAL NO. 09/064,000

Claim 382

A method for producing and integrating tissue consisting of desired soft tissue at a selected site in a body of a human patient comprising:

(a) Placing cells in said body of said human patient;

(b) Forming a bud at said selected site in said body of said human patient; and

(c) Growing said desired soft tissue which integrates itself into said body of said human patient from said bud.

Claim 383

The method of claim 382, wherein said cells are multifactorial and non-specific.

Claim 384

The method of claim 383, wherein said cells comprise stem cells.

Claim 385

The method of claim 382 further comprising forming a new artery.

Claim 386

The method of claim 383 further comprising forming a new artery.

Claim 387

The method of claim 382, wherein said soft tissue comprises mesodermal tissue.

Claim 388	The method of claim 382, wherein said soft tissue comprises an artery.
Claim 389	The method of claim 382, wherein said cells comprise stem cells.
Claim 390	The method of claim 389, wherein said soft tissue comprises an artery.
Claim 391	The method of claim 382, wherein said cells comprise pluripotent cells.
Claim 392	The method of claim 391, wherein said soft tissue comprises an artery.
Claim 393	The method of claim 391, wherein said cells comprise stem cells.
Claim 394	The method of claim 393, wherein said stem cells are multifactorial and non-specific.
Claim 395	The method of claim 382, wherein said cells are injected into said body.
Claim 396	The method of claim 382, wherein said cells are locally placed into said body.
Claim 397	The method of claim 396, wherein said cells comprise stem cells.
Claim 398	The method of claim 396, wherein said cells are injected intramuscularly.
Claim 399	The method of claim 397, wherein said stem cells are injected intramuscularly.

Claim 400	The method of claim 388 further comprising determining blood flow through said new artery.
Claim 401	The method of claim 388 further comprising observing said new artery.
Claim 402	The method of claim 399, wherein said selected site comprises a leg of said patient.
Claim 403	A method for growing and integrating tissue consisting of desired soft tissue at a selected site in a body of a human patient wherein said desired soft tissue comprises a desired artery comprising the steps of:

(a) locally injecting stem cells into said body at said selected site;

(b) forming a bud at said selected site; and

(c) growing said desired artery from said bud wherein said artery integrates itself into said body of said human patient at said selected site.

Claim 404	The method of claim 403, wherein said selected site comprises a damaged site in a leg of said patient and said stem cells are injected intramuscularly.
Claim 405	The method of claim 403, wherein said selected site comprises a damaged site in a heart of said patient and said stem cells are injected intramuscularly.

Claim 406   The method of claim 402, wherein said desired soft

tissue comprises an artery

EVIDENCE APPENDIX

ITEM NO. 12

Fourth Supplemental Declaration of Dr. Heuser
cited by Appellant as Exhibit D
in the Response filed November 28, 2007

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Re Application of: James P. Elia)

Serial No.: 09/064,000)

Filed: April 21, 1998)

For: METHOD FOR GROWTH)
     OF SOFT TISSUE)

) Group Art Unit: 1646
)
) Examiner: Elizabeth C. Kemmerer
)
)
)
)

FOURTH SUPPLEMENTAL DECLARATION
OF RICHARD HEUSER, M.D., F.A.C.C., F.A.C.P.

I Richard Heuser declare as follows:

1.   I have offices at 500 West Thomas Road, Suite 900, Phoenix, Arizona 85013.

2.   My Curriculum Vitae was attached as Exhibit A to my Declaration of November 11, 2004.   Paragraph 3 of my Declaration and my Supplemental Declaration of February 15, 2005 provide additional information regarding my background and experience.

3.   I have read and understood the disclosures of the above-referenced patent application at page 20, line 10 through page 21, line 15; at page 37, lines 19-25; at page 44, line 19 through page 46, line 16; and at page 47, line 22 through page 48, line 15. A copy of such disclosures was attached to my Third Supplemental Declaration as Exhibit A.

I have also read and understood additional disclosures of the above-referenced patent application at page 33, lines 8-10; page 40, line 20 through page 43 line 3; page 44, lines 12 and 13; page 48, lines 13-15; page 49, lines 18-22; page 53, line 1 through page 56, line 25; and page 62, lines 1-10;. A copy of such additional disclosures was attached to my Third Supplemental Declaration as Exhibit B.

4.   I note that the disclosures referenced in above Paragraph 3 relate to using a growth factor for promoting the growth of soft tissue, and more specifically, to a method of using a cell, such as a stem cell, to grow soft tissue, such as an artery.

5.   I have read and understood the claims set forth in the attached Fourth Supplemental Declaration Exhibit A and have been informed that such claims will be concurrently presented in the above-referenced patent application with this Fourth Supplemental Declaration.

6.   Based upon above Paragraphs 3-5, it is my opinion that introducing a growth factor, including cells, and more specifically, stem cells, in the body of a human patient will predictably result in the growth of soft tissue, such as an artery, which will integrate itself into pre-existing tissue of the body thereby forming a unified whole.

7.   Based upon above Paragraphs 3-5, it is my opinion that one skilled in the medical arts, armed with the knowledge in such paragraphs, would be able to practice the method set forth in Exhibit A without need for resorting to undue experimentation.

8.   Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: _____

_____
Richard Heuser

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FOURTH SUPPLEMENTAL DECLARATION

EXHIBIT A

CLAIMS

EXHIBIT A

CLAIMS
APPLICATION SERIAL NO. 09/064,000

Claim 403 — A method for growing and integrating tissue consisting of desired soft tissue at a selected site in a body of a human patient wherein said desired soft tissue comprises a desired artery comprising the steps of:

(a) locally injecting stem cells into said body at said selected site;

(b) forming a bud at said selected site; and

(c) growing said desired artery from said bud wherein said artery integrates itself into said body of said human patient at said selected site.

Claim 404 — The method of claim 403, wherein said selected site comprises a damaged site in a leg of said patient and said stem cells are injected intramuscularly.

Claim 405 — The method of claim 403, wherein said selected site comprises a damaged site in a heart of said patient and said stem cells are injected intramuscularly.

Claim 407 — The method of claim 403, wherein said stem cell comprises a living stem cell harvested from bone marrow.

Claim 408

The method of claim 407, wherein said bone marrow is from said patient.

Claim 409

The method of claim 403, wherein said stem cell comprises a living stem cell harvested from blood.

Claim 410

The method of claim 409, wherein said blood is from said patient.

Claim 411

The method of claim 403 further comprising determining blood flow through said desired artery.

Claim 412

The method of claim 403 further comprising observing said desired artery.

EVIDENCE APPENDIX

ITEM NO. 8

**Third Supplemental Declaration of Dr. Heuser
(originally filed in co-pending application Serial No. 10/179,589) and
cited by Appellant as Exhibit A in the Letter filed May 25, 2007**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: James P. Elia)
)   Group Art Unit: 1646
Serial No.: 10/179,589)
)   Examiner: Daniel C. Gamett
Filed: June 25, 2002)
)
For: METHOD FOR GROWING)
     HUMAN ORGANS AND)
     SUBORGANS)

THIRD SUPPLEMENTAL DECLARATION
OF RICHARD HEUSER, M.D., F.A.C.C., F.A.C.P.

I Richard Heuser declare as follows:

1.   I have offices at 500 West Thomas Road, Suite 900, Phoenix, Arizona 85013.

2.   My Curriculum Vitae was attached as Exhibit A to my Declaration of November 16, 2004. Paragraph 3 of my Declaration and my Supplemental Declaration of February 15, 2005 provide additional information regarding my background and experience.

3.   I have read the Examiner's criticism contained in paragraph 11, commencing on page 7 and ending on page 9 of the March 7, 2007 Office Action regarding the conversion of dosages of plasmid cDNA to dosages of cells. Such paragraph is set forth in Third Supplemental Exhibit A attached hereto. Specifically, I note the Examiner's criticism bridging pages 7 and 8 regarding the above-mentioned conversion that:

...one of skill in the art would never think to attempt such an extrapolation. The unsound scientific basis for the conversion of plasmid DNA to cellular equivalents would be obvious to anyone trained in molecular biology.

4.   I have read and understood the disclosures of the above-referenced patent application at page 4, line 1 through page 5, line 14; at page 13, lines 3-10; at page 22, line 5 through page 24, line 15; and at page 26, line 3 through page 27, line 3. A copy of such disclosures is attached hereto as Third Supplemental Declaration Exhibit B.

I have also read and understood additional disclosures of the above-referenced patent application at page 9, lines 14-16; page 17, line 1 through page 20 line 8; page 21, lines 23 and 24; page 27, lines 1-3; page 28, lines 12-16; page 32, line 20 through page 39, line 19; and page 44, lines 8-17. A copy of such additional disclosures is attached hereto as Third Supplemental Declaration Exhibit C.

5.   I have read and understood Applicant's conversion for dosages of plasmid cDNA to equivalent corresponding dosages of cells set forth in attached Third Supplemental Exhibit D as it relates to Examples 18 and 17 of the specification, which are contained in Third Supplemental Declaration Exhibit C.

6.   In my opinion, the Examiner's criticism specifically delineated in Paragraph 3 above is not credible. Contrary to the Examiner's opinion, studies involving conversion of the average (mean) content of nucleic acids per cell in human marrow cells have been routinely conducted and accepted by skilled scientists for over 50 years. Three (3) publications illustrating the use of such well known conversion are included in the attached Third Supplemental Declaration Exhibit E. Note that in two of

the publications, typical conversion results are set forth in tables, thereby eliminating the necessity to perform the actual calculation. Obviously, a sound scientific basis exists in the medical art for such conversions.

Further, those skilled in the art understand that DNA content is substantially consistent from tissues of any given species. Consequently, a skilled medical person relying on sound scientific bases at the time of the present invention would reasonably have understood how to extrapolate plasmid DNA to cells on a weight basis. Applicant's use of 40 pg as an average weight for nucleic acids in a human cell is fairly representative. Thus, I find Applicant's conversion set forth in the attached Third Supplemental Declaration Exhibit D to be consistent with the extrapolations set forth above and commonly used and relied upon by skilled persons in the medical art. Accordingly, the dosages specified in Examples 18 and 17 are sufficient to enable a person skilled in the medical art to convert dosages of plasmid DNA to corresponding dosages of genomic DNA within the context of Applicant's disclosed invention.

7.   Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date:_____   _____

                                    Richard Heuser

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THIRD SUPPLEMENTAL
DECLARATION

EXHIBIT A

MARCH 8, 2007
OFFICE ACTION
Paragraph 11, pages 7-9

11.   Applicant admits on page 9 that Examples 17-19 employ nucleic acids, but asserts that one skilled in the art reading the specification, which teaches that cells, i.e., stem cells (BMC's) possess equivalent activity to genes (nucleic acids) and other genetic material in forming a new artery (i.e., promote morphogenesis of an organ—artery), would be able to easily extrapolate the number on a weight basis of mononuclear cells required to obtain equivalent results. According to the method for extrapolation provided in the footnote to pages 10-11, 250 µg of plasmid DNA (an amount described in Examples 17 and 18) divided by 40 pg, (asserted to be is the average DNA content of a cell; the species of cell is not disclosed) equals 6.25×10^6, and therefore the Examples 17 and 18 instruct the skilled artisan to use 6.25×10^6 cells. This argument is not persuasive for several reasons. First, this method of converting plasmid DNA to cell equivalents is not included in the specification as filed. This is important because one of skill in the art would never think to attempt such an extrapolation. The unsound scientific basis for the conversion of µg of plasmid DNA to cellular equivalents would be obvious to anyone trained in molecular

- A1 -

biology. One basic assumption of the recited conversion is that the 40 pg of cellular DNA comprises the same gene dosage a purified plasmid DNA. Every molecule of the postulated plasmid DNA comprises a copy of the VEGF cDNA. In contrast, VEGF coding sequences would comprise but one of 30-40 thousand genes in genomic DNA (at the time of filing, it was widely believed that the human genome comprised 100,000 genes). Therefore, one of skill in the art at the time of filing would not expect plasmid DNA and genomic DNA to be comparable on a per weight basis. Applicant's argument seems to view the living cell as little more than a container for DNA. The expression of the recombinant cDNA would be under control of the limited number of enhancer and promoter elements in the plasmid, as opposed the native control elements with the genome. Therefore, even equivalent gene doses would not be expected to yield equivalent amounts of gene product with a plasmid as opposed to a cell. Applicant's argument seems to view the living cell as little more than a container for DNA. Delivery of the genes to a target as recombinant DNA as opposed to native genes within a living cell are technically different processes; there is no basis for using one to guide the other. For example, with DNA one is concerned with chemical stability, efficiency of uptake, stable retention, and subsequent expression of the injected molecule into target cells, whereas with cells separate issues of formation of effective attachment to ECM and neighboring cells, short- and long-term viability, and responses to environmental cues arise. As evidence, one need look no further than the US Patent classification system. Methods of *in vivo* treatments involving whole live cells as opposed to nucleic acids are separately classified: class 424 subclass 93.1 (cells); class 514, subclass 44 (polynucleotides). These separate classifications indicate a different status in the art such that it is well known that cell therapy and gene therapy are not obvious variants of one

- A2 -

another. Therefore, contrary to Applicants assertion on page 9, the specification does not

describe any dosage of cells to use to promote artery growth.

THIRD SUPPLEMENTAL DECLARATION

EXHIBIT B

DISCLOSURES

EXHIBIT B

DISCLOSURES
APPLICATION SERIAL NO. 10/179,589

PAGE 4, LINE 1 – PAGE 5, LINE 14

Growth factors can be utilized to induce the growth of "hard tissue" or bone and "soft

tissues" like ectodermal and mesodermal tissues. As used herein, the term growth factor

encompasses compositions and living organisms which promote the growth of hard tissue, such

as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic

matter. The compositions can be genetically produced or manipulated. The living organisms

can be bacteria, viruses, or any other living organism which promote tissue growth. By way of

example and not limitation, growth factors can include platelet-derived growth factor (PDGF),

epidermal growth factor (EGF), fibroblast growth factor (acidic/basis (FGF a,b), interleukins

(IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating

factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF),

bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-

recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate,

be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-

assembles into a nanometer internal diameter pore to build a selective lipid body. Various

enzymes can be utilized for the synthesis of peptides which contain amino acids that control

three-dimensional protein structure and growth. Growth factors can be applied in gels or other

carriers which regulate the rate of release of the growth factors and help maintain the growth

factors and the carrier, at a desired location in the body. Time release capsules, granules, or

other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor an also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which an often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 13, LINES 3-10

Multifactorial and nonspecific cells (such as stem cells and germinal cells) can provide the necessary *in vivo* and *in vitro* cascade of genetic material once an implanted master control gene's transcription has been activated. Likewise, any host cell, cloned cell, cultured cell, or cell would work. Genetic switches (such as the insect hormone ecdysone) can be used to control genes inserted into humans and animals. These gene switches can also be used in cultured cells or other cells. Gene switches govern whether a gene is on or off making possible precise time of gene activity.

PAGE 22, LINE 5 – PAGE 24, LINE 15

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in connection with any of the implant techniques of the invention, it is understood that a cell nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated

- B3 -

(taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth

factors (or other genetic material) are placed. Once the new muscle and blood vessels have grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

PAGE 26, LINE 3 – PAGE 27, LINE 3

Organs and/or tissues can be formed utilizing the patient's own cells. For example, a skin cell(s) is removed from the intraoral lining of a cheek. The cell is genetically screened to identify DNA damage or other structural and/or functional problems. Any existing prior art genetic screening technique can be utilized. Such methods can utilize lasers, DNA probes, PCR, or any other suitable device,. If the cell is damaged, a healthy undamaged cell is, if possible, identified and selected. If a healthy cell cannot be obtained, the damaged cell can be repaired by excision, alkylation, transition, or any other desired method. A growth factor(s) is added to the cell to facilitate dedifferentiation and then redifferentiation and morphogenesis into an organ or function specific tissue. Any machine known in the art can be used to check the genetic fitness of the organ and its stage of morphogenesis. A cell nutrient culture may or may not be utilized depending on the desired functional outcome (i.e., growth of an artery, of pancreatic Islet cells, of a heart, etc.) or other circumstances. Replantation can occur at any appropriate stage of

morphogenesis. The foregoing can be repeated without the patient's own cells if universal donor cells such as germinal cells are utilized. Germinal cells do not require a dedifferentiation. They simply differentiate into desired tissues or organs when properly stimulated. Similarly, the DNA utilized in the foreign procedure can come from the patient or from any desired source.

During reimplantation one of the patient's own cells is returned to the patient. During implantation, a cell not originally obtained from the patient is inserted on or in the patient.

In the example above, if germinal cells (and in some cases, stem cells) are utilized, a direct differentiation and morphogenesis into an organ can occur *in vivo, ex vivo,* or *in vitro.*

THIRD SUPPLEMENTAL DECLARATION

EXHIBIT C

ADDITIONAL DISCLOSURES

EXHIBIT C

DISCLOSURES
APPLICATION SERIAL NO. 10/179,589

PAGE 9, LINES 14-16

Morphogenesis or morphogenetics is the origin and evolution of morphological characters and is the growth and differentiation of cells and tissues during development.

PAGE 17, LINE 1 – PAGE 20, LINE 8

EXAMPLE 10

MSX-1 and MSX-2 are the homeobox genes that control the generation and growth of a tooth. A sample of skin tissue is removed from the patient and the MSX-1 and MSX-2 homeobox gene(s) are removed from skin tissue cells. The genes are stored in an appropriate nutrient culture medium.

BMP-2 and BMP-4 growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 and 102 degrees F.

MSX-1 and MSX-2 transcription factors are obtained which will initiate the expression of the MSX-1 and MSX-2 homeobox genes.

The MSX-1 and MSX-2 transcription factors, BMP-2 and MBP-4 bone morphogenic proteins, and MSX-1 and MSX-2 genes are added to the nutrient culture medium along with the living stem cells.

EXAMPLE 11

Example 10 is repeated except that the transcription factors bind to a receptor complex in the stem cell nucleus.

EXAMPLE 12

Example 10 is repeated except that the MSX-1 and MSX-2 transcription factors are not utilized. The transcription of the MSX-1 and MSX-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 13

Example 10 is repeated except that the stem cells are starved and the transcription of the MSX-1 and MSX-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 14

WT-1 and PAX genes are obtained from a sample of skin tissue removed from the patient. The genes are stored in an appropriate nutrient culture medium. PAX genes produce PAX-2 and other transcription factors.

BMP-7 and other kidney related BMP growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

The WT-1 and PAX genes, and BMP-7 and other kidney BMPS are added to the nutrient culture medium along with the living stem cells.

A primitive kidney germ is produced. The kidney germ is transplanted in the patient's body near a large artery. As the kidney grows, its blood supply will be derived from the artery.

EXAMPLE 15

The Aniridia gene is obtained from a sample of skin tissue removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

The Aniridia transcription factor and growth factor and the Aniridia gene are added to the nutrient culture medium along with the living stem cells.

A primitive eye germ is produced. The eye germ is transplanted in the patient's body near the optic nerve. As the eye grows, its blood supply will be derived from nearby arteries.

EXAMPLE 16

The Aniridia gene is obtained from a sample of skin tissue removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained and added to the nutrient culture medium.

An eye germ develops. A branch of the nearby maxillary artery is translocated to a position adjacent the eye germ to promote the development of the eye germ. The eye germ matures into an eye which receives its blood supply from the maxillary artery.

The term "cell nutrient culture" as used herein can include any or any combination of the following: the extracellular matrix; conventional cell culture nutrients; and/or, a cell nutrient such as a vitamin. As such, the cell nutrient culture can be two-dimensional, three-dimensional, or simply a nutrient, and is useful in promoting the processes of cellular dedifferentiation, redifferentiation, differentiation, growth, and development.

PAGE 21, LINES 23– 24

An organ, as used herein, consists of two or more kinds of tissues joined into one structure that has a certain task.

PAGE 27, LINES 1–3

In the example above, if germinal cells (and in some cases, stem cells) are utilized a direct differentiation and morphogenesis into an organ can occur *in vivo*, *ex vivo*, or *in vitro*.

PAGE 28, LINES 12-16

Avascular necrosis can be corrected with the insertion of a gene(s) and/or growth factor or other genetic material in the body. For example, avascular necrosis is diagnosed near a joint space. VEGF or BMP genes, or VEFG or BMP growth factors produced by VEFG or BMP genes, respectively, or any other desired genetic based material can be inserted to regrow blood vessels and/or bone.

PAGE 32, LINE 20 – PAGE 39, LINE 19

EXAMPLE 17

A 36-year old Caucasian male experiences pain in his left leg. A medical examination reveals a damaged one-inch long section of a large artery in his left leg. The examination also reveals that this damaged section of the artery is nearly completely clogged with plaque and that the wall of the artery is weakened. The weakening in the arterial wall makes attempting to clean out the artery risky and also makes it risky to attempt to insert a stent in the artery.

Recombinant cDNA encoded to combine with a cell ribosome to produce the human growth factor VEGF is assembled into a eukaryotic expression plasmid. The recombinant

cDNA is from cDNA libraries prepared from HL60 leukemia cells and is known to cause the growth of arteries. The plasmid is maintained at a room temperature of 76 degrees F.

The clones are placed in 1.0 milliliters of a normal saline carrier solution at a room temperature of 76 degrees F. to produce a genetic carrier solution. The genetic carrier solution contains about 250 ug of the cDNA clones. A nutrient culture can, if desired, be utilized in conjunction with or in place of the saline carrier. Each clone is identical. If desired, only a single clone can be inserted in the normal saline carrier solution. The saline carrier solution comprises 0.09% by weight sodium chloride in water. A saline carrier solution is selected because it will not harm the DNA clone.

Two sites are selected for injection of the genetic carrier solution. While the selection of sites can vary as desired, the sites are selected at the lower end (the end nearest the left foot of the patient) of the damaged section of the artery so that the new arterial section grown, can, if necessary, be used to take the place of the damaged section of the artery in the event the damaged section is removed.

The first site is on the exterior wall of the artery on one side of the lower end of the damaged section of the artery. A containment system is placed at the first site.

The second site is inside the wall of the artery on the other side of the lower end of the artery.

The genetic carrier solution is heated to a temperature of 98.6 degrees F. 0.25 milliliters of the genetic carrier solution is injected into the containment system at the first site. 0.25 milliliters of the genetic carrier solution is injected at the second site inside the wall of the artery. Care is taken to slowly inject the genetic carrier solution to avoid entry of the solution into the artery such that blood stream will carry away the cDNA in the solution.

After two weeks, an MRI is taken which shows the patient's leg artery. The MRI reveals new growth at the first and second sites.

After four weeks, another MRI is taken which shows the patient's leg artery. The MRI shows that (1) at the first site, a new artery is growing adjacent the patient's original leg artery, and (2) at the second site, a new section of artery is growing integral with the original artery, i.e., at the second site the new section of artery is lengthening the original artery, much like inserting a new section of hose in a garden hose concentric with the longitudinal axis of the garden hose lengthens the garden hose.

After about eight to twelve weeks, another MRI is taken which shows that the new artery growing adjacent the patient's original artery has grown to a length of about one inch and has integrated itself at each of its ends with the original artery such that blood flows through the new section of artery. The MRI also shows that the new artery at the second site has grown to a length of one-half inch.

In any of the examples of the practice of the invention included herein, cell nutrient culture can be included with the gene, the growth factor, the extracellular matrix, or the environmental factors.

In any of the examples of the practice of the invention included herein, the concept of gene redundancy can be applied. For example, the Examples 1 to 14 concerning a tooth list the genes MSX-1 and MSX-2. These genes differ by only two base pairs. Either gene alone may be sufficient. A further example of redundancy occurs in growth factors. Looking at the Examples 10 to14, BMP4 or BMP2 alone may be sufficient. Redundancy can also be utilized in connection with transcription factors, extracellular matrices, environmental factors, cell nutrient culture, physiological nutrient cultures, vectors, promoters, etc.

One embodiment of the invention inserts genetic material (gene, growth factor, ECM, etc.) into the body to induce the formation of an organ. Similar inducing materials *ex vivo* into or onto a living cell in an appropriate physiological nurturing environment will also induce the growth of an organ. The VCSEL laser allows early detection in a living cell of a morphogenic change indicating that organ formation has been initiated. With properly time transplantation, organ growth completes itself.

During the *ex vivo* application of the invention, a gene and/or growth factor is inserted into a cell or a group of cells; an ECM or environmental factor(s) are placed around and in contact with a cell or group of cells; or, genetic material is inserted into a subunit of a cell to induce organ growth. An example of a subunit of a cell is an enucleated cell or a comparable artificially produced environment. In *in vivo* or *ex vivo* embodiments of the invention to induce the growth of an organ, the genes, growth factors, or other genetic material, as well as the environmental factors or cells utilized, can come from any desired source.

EXAMPLE 18

Genetically produced materials are inserted in the body to cause the body to grow, reproduce, and replace *in vivo* a clogged artery in the heart. This is an example of site-specific gene expression. A plasmid expression vector containing an enhancer/promoter is utilized to aid in the transfer of the gene into muscle cells. The enhancer is utilized to drive the specific expression of the transcriptional activator. After the enhancer drives the expression of the transcriptional activator, the transcriptional activator transactivates the muscle/artery genes. Saline is used as a carrier. Cardiac muscle can take up naked DNA injection intramuscularly.

Injecting plasmid DNA into cardiac (or skeletal) muscle results in expression of the transgene in cardiac myocytes for several weeks or longer.

Readily available off-the-shelf (RAOTS) cDNA clones for recombinant human VEFG165, isolated from cDNA libraries prepared from HL60 leukemia cells, are assembled in a RAOTS expression plasmid utilizing 736bp CMV promoter/enhancer to drive VEGF expression. Other RAOTS promoters can be utilized to drive VEGF expression for longer periods of time. Other RAOTS recombinant clones of angiogenic growth factors other than VEGF can be utilized, for example, fibroblast growth factor family, endothelial cell growth factor, etc. Downstream from the VEGF cDNA is an SV40 polyadenylation sequence. These fragments occur in the RAOTS pUC118 vector, which includes an Escherichia coli origin of replication and the Beta lactamase gene for ampicillin resistance.

The RAOTS construct is placed into a RAOTS 3 ml syringe with neutral pH physiologic saline at room temperature (or body temperature of about 73 degrees C). The syringe has a RAOTS 27 gauge needle.

Access to the cardiac muscle is gained by open-heart surgery, endoscopic surgery, direct injection of the needle with incision, or by any other desired means. The cardiac muscle immediately adjacent a clogged artery is slowly injected with the RAOTS construct during a five second time period. Injection is slow to avoid leakage through the external covering of muscle cells. About 0.5 ml to 1.0 ml (milliliter) of fluid is injected containing approximately 500 ug phVEGF165 in saline (N=18). The readily available off-the-shelf cDNA clones cause vascular growth which automatically integrates itself with the cardiac muscle. Anatomic evidence of collateral artery formation is observed by the 30th day following injection to the RAOTS construct. One end of the artery integrates itself in the heart wall to receive blood from the heart.

The other end of the artery branches into increasingly smaller blood vessels to distribute blood into the heart muscle. Once the growth of the new artery is completed, the new artery is left in place in the heart wall. Transplantation of the new artery is not required.

Blood flow through the new artery is calculated in a number of ways. For example, Doppler-derived flow can be determined by electromagnetic flowmeters (using, for example, a Doppler Flowmeter sold by Parks Medical Electronic of Aloha, Oregon) both *in vitro* and *in vivo*. Also, RAOTS angiograms or any other readily available commercial devices can be utilized.

VEGF gene expression can be evaluated by readily available off-the-shelf polymerase chain reaction (PCR) techniques.

If controls are desired, the plasmid pGSVLacZ containing a nuclear targeted Beta-galactosidase sequence coupled to the simian virus 40 early promoter can be used. To evaluate efficiency, a promoter-matched reporter plasmid, pCMV Beta (available from Clontech of Palo Alto, California), which encodes Beta-galactosidase under control of CMV promoter/enhancer, can be utilized. Other RAOTS products can be utilized if desired.

EXAMPLE 19

A patient, a forty-year old African-American female in good health, has been missing tooth number 24 for ten years. The space in her mouth in which her number 24 tooth originally resided is empty. All other teeth except tooth number 24 are present in the patient's mouth. The patient desires a new tooth in the empty "number 24" space in her mouth.

A full thickness mucoperiosteal flap surgery is utilized to expose the bone in the number 24 space. A slight tissue reflection into the number 23 tooth and number 25 tooth areas is carried out to insure adequate working conditions.

A Midwest Quietair handpiece (or other off-the-shelf handpiece) utilizing a #701 XXL bur (Dentsply Midwest of Des Plaines, Illinois) (a #700, #557, #558, etc. bur can be utilized if desired) is used to excavate an implant opening or site in the bone. The implant opening is placed midway between the roots of the number 23 and number 25 teeth. The opening ends at a depth which is about fifteen millimeters and which approximates the depth of the apices of the roots of the number 23 and number 25 teeth. Care is taken not to perforate either the buccal or lingual wall of the bone. In addition, care is taken not to perforate or invade the periodontal ligament space of teeth numbers 23 and 25.

An interrupted drilling technique is utilized to avoid overheating the bone when the #701XXL bur is utilized to form the implant opening. During a drilling sequence, the drill is operated in five-second increments; and the handpiece is permitted to stall. Light pressure and a gentle downward stroke are utilized.

PAGE 44, LINES 8–17

EXAMPLE 35

Example 17 is repeated except that the patient is a 55-year old Caucasian male, and the genetic carrier solution is injected into two sites in the coronary artery of the patient. The first site is on the exterior wall on one side of the artery. The second site is inside the wall of the artery on the other side of the artery. A section of the artery is damaged, is partially blocked, and has a weakened wall. The first and second sites are each below the damaged section of the

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artery. Similar results are obtained, i.e., a new section of artery grows integral with the original artery, and a new section of artery grows adjacent the original artery. The new section of artery has integrated itself at either end with the original artery so that blood flows through the new section of artery.

THIRD SUPPLEMENTAL
DECLARATION

EXHIBIT D

CONVERSION

EXHIBIT D

CONVERSION

The conversion for dosages of nucleic acids to corresponding dosages of cells was conducted as follows. Examples 18 and 17 specified dosages of 500 micrograms (ug) and 250 ug, respectively. The weight of nucleic acids of an average cell was considered to equal 40 picograms (pg). The described dosages of 250 and 500 ug when converted to pg by multiplying by 10^6 equals 250×10^6 pg and 500×10^6 pg. Since nucleic acids of an average cell have an average weight of 40 pg, a conversion is made by dividing 250×10^6 and 500×10^6 by 40 to arrive at the equivalent cell dosages, which are 6.25×10^6 and 12.5×10^6, respectively.

THIRD SUPPLEMENTAL DECLARATION

ENTITLED

PUBLICATIONS (5)

Studies on the Average Content of Nucleic Acids in Human Marrow Cells. By J. N. DAVIDSON, I. LESLIE and J. C. WHITE. (*From the Department of Biochemistry, University of Glasgow, and the Department of Pathology, Postgraduate Medical School of London*)

In extension of previously reported analyses of the deoxyribonucleic acid phosphorus (DNAP) and ribonucleic acid phosphorus (RNAP) content of aspirated human bone marrow (Davidson, Leslie & White, 1947, 1948), we now report a modification involving enumeration of the nucleated cell content of the samples analysed. Results are expressed in terms of DNAP and RNAP per cell (Table 1), and are average values for the growing and adult cell populations of the analysed samples. The recent results of Vendrely & Vendrely (1948, 1949) and of Mirsky & Ris (1949) suggest a striking constancy in the DNAP content of normal cell nuclei from the tissues of any given species, and our figures for DNAP are of the same order as those quoted by the Vendrelys for human liver nuclei.

There is no significant difference between the means for the normal and the leukaemic series, either as a whole, or considering only acute leukaemia prior to therapy.

A small series of 6 cases of iron-deficiency anaemia has not shown significant variation of the mean DNAP and RNAP per cell from normal.

Results obtained from cases of pernicious and other megaloblastic anaemias are shown in Tables 2 and 3.

It must be noted clearly that the group under therapy cannot be considered as returned to normal, either as regards blood picture, marrow cytology or adequacy of therapy. The significant fall in RNAP from that in the group prior to therapy parallels the general increase in maturity of the marrow under therapy. Cases fully treated and returned to normal are under investigation.

Table 1

Normal human marrow

Values of Nucleic Acid Phosphorus (NAP) in $\mu g. \times 10^{-7}$ per cell

	DNAP 18 obs. on 16 individuals	RNAP 20 obs. on 18 individuals	Ratio RNAP/DNAP
Mean	8·54	6·33	0·75
s.e. of obs.	2·89	3·03	0·326
Observed range	4·0–15·0	2·1–13·5	0·43–1·9

Marrow from cases of leukaemia of various types, before and during therapy

	28 obs. on 15 cases	24 obs. on 12 cases	
Mean	8·75	7·59	0·90
s.e. of obs.	3·05	3·72	0·30
Observed range	3·9–17·4	2·0–17·4	0·3–1·8

Table 2. Cases of pernicious anaemia and other megaloblastic anaemias

NAP in $\mu g. \times 10^{-7}$ per cell

		DNAP	RNAP	Ratio DNAP/RNAP
Group as a whole	Mean	28 obs. on 12 cases 12·6	10·9	28 obs. on 13 cases 0·87
	s.e.	4·56	5·03	0·27
	Observed range	6·6–22·8	2·3–25·1	0·35–1·5
Group prior to therapy	Mean	12 obs. on 12 cases 12·57	11 obs. on 11 cases 13·38	12 obs. on 12 cases 1·06
	s.e.	4·17	5·19	0·249
	Observed range	8·1–22·8	7·5–25·1	0·69–1·5
Group during the course of therapy	Mean	17 obs. on 8 cases 12·63	15 obs. on 8 cases 9·09	16 obs. on 9 cases 0·73
	s.e.	4·36	4·21	0·198
	Observed range	6·6–18·8	2·3–17·6	0·35–1·0

Table 3. t test of significance between means

		DNAP	RNAP	Ratio RNAP/DNAP
Megaloblastic series as a whole compared with normal series	P Degrees of freedom	<0·001 44 Highly significant	<0·001 44 Highly significant	0·2–0·1 46 Not significant
Megaloblastic series before therapy compared with normal	P Degrees of freedom	0·01–0·001 28 Highly significant	<0·001 29 Highly significant	0·01–0·001 30 Highly significant
Megaloblastic series during therapy compared with normal	P Degrees of freedom	0·01–0·001 33 Highly significant	0·05–0·02 33 Significant	0·8–0·7 34 Not significant
Megaloblastic series before and during therapy compared	P Degrees of freedom	0·7–0·6 27 Not significant	0·05–0·02 24 Significant	<0·001 26 Highly significant

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Fluoroacetate Poisoning and 'Jamming' of the Tricarboxylic Acid Cycle; Mode of Action of an 'Active' Fluoro Compound Synthesized via this Cycle. By P. BUFFA, W. D. LOTSPEICH, R. A. PETERS and R. W. WAKELIN. (Department of Biochemistry, University of Oxford)

So far no isolated enzyme has been inhibited by fluoroacetate. The hypothesis has been advanced by Liébecq & Peters (1949) (see also Martius, 1949) that the inhibition of citrate oxidation, occurring also in vivo (Buffa & Peters, 1949), is due to the 'jamming' effect of an enzymically synthesized fluoro-tricarboxylic acid in the Krebs tricarboxylic acid cycle. In support of this hypothesis, Buffa, Peters & Wakelin (1950) have isolated, from guinea-pig kidney homogenates treated with fluoroacetate, a tricarboxylic fraction, which is 'active' in preventing disappearance of added citrate. This active fraction is mainly citrate; it contains no fluoro-acetate, but there is present a small amount of a F-compound which is chromatographically inseparable from the tricarboxylic acids.

We have tried to find the exact point of inhibition in the enzymes of the tricarboxylic acid cycle by determining the effect of the 'active' fractions upon aconitase (Johnson, 1939), isocitric dehydrogenase (Adler, Euler, Günther & Plass, 1939) and oxalo-succinic decarboxylase (Ochoa & Weiss-Tabori, 1948), obtained from rat and pig heart tissue. Tables 1, 2 and 3 show that the results were negative, even when amounts of 'active' fraction were used 80 times larger than those inhibiting citrate disappearance in the kidney homogenates.

All the evidence from experiments in vivo and in vitro (? mitochondrial homogenates) points to inhibition by the 'active' compound at either the

aconitase or isocitric dehydrogenase stage. Hence, we are led to the conclusion that the complete system has properties not present in its isolated enzyme components. Whether these be due to factors of organization or to missing components must be decided by further work.

Table 1. Rat heart aconitase

Time (min.) ...	Citric acid (μmol.)	
Additions:	0	60
cis-Aconitate (5 μmol.)	0·21	3·90
cis-Aconitate + 'active' fraction	0·08	3·96
Citrate (5 μmol.)	4·90	4·34
Citrate + 'active' fraction	5·27	4·38

Table 2. Pig heart isocitric dehydrogenase

	$E_{340 m\mu}$ (max. value)
DL-isocitrate only	0·076–0·065
Same + 'active' fraction	0·075
Same + p-chloromercuribenzoic acid 1·33 × 10⁻³ M	0·004

Table 3. Pig heart oxalosuccinic decarboxylase

(CO₂ evolution from 10 μmol. oxalosuccinate in 30 min. at 13·5°C. Net values)

	CO_2 (μl.)
Enzyme alone	83
Enzyme + 'active' fraction	76
Enzyme + DL-isocitrate (control)	14

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Nucleic Acids

Content and Distribution

Nucleic acids in an average human cell

DNA	
Coding sequences	~6 pg/cella
Number of genes	3% of genomic DNA
Active genes	0.5 1.0 x 10^5
	1.5 x 104
Total RNA	
rRNAs	~10 50 pg/cellb
tRNAs, snRNAs, and low mol. wt. RNA	80 85% of total RNA
mRNAs	15 20% of total RNA
nuclear RNA	1 5% of total RNA
Ratio of DNA:RNA in nucleus	~14% of total RNA
Number of mRNA moleculesc	~2:1
Number of different mRNA species	0.2 1.0 x 10^6
Low abundance mRNA (5 15 copies/cell)	1.0 3.4 x 10^4
Intermediate abundance mRNA (200 400 copies/cell)	11,000 different messages
High abundance mRNA (12,000 copies/cell)	500 different messages
Abundance of each message for:	<10 different messages
Low abundance mRNA (5 15 copies/cell)	<0.004% of total mRNA
Intermediate abundance mRNA (200 400 copies/cell)	<0.1% of total mRNA
High abundance mRNA (12,000 copies/cell)	3% of total mRNA

a   30 – 60 μg/ml blood for human leukocytes.

b   1 – 5 μg/ml blood for human leukocytes.

c   Average size of mRNA molecule = 1930 bases.

RNA content of cells in culture

Type of cell	Total RNA (mRNA (μg/107 cells)	mRNA (μg/107 cells)
NIH/3T3 cells	75 200	1.5 4.0
HeLa cells	100 300	2 6
CHO cells	200 400	3 6

<< >>

Table of Content

UMRECHNUNGSTABELLEN

I. Conversiontable

Molecular weight (daltons)	1 μg	1 nmole
100	10 nmoles or 6 x 10^{15} molecules	0.1 μg
1,000	1 nmole or 6 x 10^{14} molecules	1 μg
10,000	100 pmoles or 6 x 10^{13} molecules	10 μg
20,000	50 pmoles or 3 x 10^{13} molecules	20 μg
30,000	33 pmoles or 2 x 10^{13} molecules	30 μg
40,000	25 pmoles or 1.5 x 10^{13} molecules	40 μg
50,000	20 pmoles or 1.2 x 10^{13} molecules	50 μg
60,000	17 pmoles or 10^{13} molecules	60 μg
70,000	14 pmoles or 8.6 x 10^{12} molecules	70 μg
80,000	12 pmoles or 7.5 x 10^{12} molecules	80 μg
90,000	11 pmoles or 6.6 x 10^{12} molecules	90 μg
100,000	10 pmoles or 6 x 10^{12} molecules	100 μg
120,000	8.3 pmoles or 5 x 10^{12} molecules	120 μg
140,000	7.1 pmoles or 4.3 x 10^{12} molecules	140 μg
160,000	6.3 pmoles or 3.8 x 10^{12} molecules	160 μg
180,000	5.6 pmoles or 3.3 x 10^{12} molecules	180 μg
200,000	5 pmoles or 3 x 10^{12} molecules	200 μg

II. Some useful nucleotide dimensions

1 cm of DNA ~ 3 x 10^6 nucleotides

Organism	Base pairs/ haploid genome	Base pairs/ diploid genome	Length/cell	Mass

Human	3×10^9	6×10^9	2 meters (diploid)	6 pg
Fly	1.65×10^8	3.3×10^8	100 cm (diploid)	0.3 pg
Yeast	1.35×10^7	2.7×10^7	10 cm (dolploid)	0.03 pg
E. coli	4.7×10^6	-	1.5 cm (diploid)	0.0045 pg
SV40	5×10^3	-	1.7 nm	0.000006 pg

III. Some useful cell dimensions

Organism	Dimensions	Volume
S. cerevisiae	5 µm	66 µm³
S. pombe	2 x 7 µm	22 µm³
Mammalian cell	10-20 µm	500-4,000 µm³
E. coli	1 x 3 µm	2 µm³
Mammalian mitochondrion	1 µm	0.5 µm³
Mammalian nucleus	5-10 µm	66-500 µm³
Plant chloroplast	1 x 4 µm	3 µm³
Bacteriophage lambda	50 nm (head only)	6.6×10^{-5} µm³
Ribosome	30 nm diameter	1.4×10^{-5} µm³
Globular monomeric protein	5 nm diameter	6.6×10^{-8} µm³

III. Some useful concentrations

Total cell protein concentration   Detergent soluble protein = 1-2 mg/ 10^7 mammalian cells or 100-200 mg/ ml for soluble proteins only

Specific protein concentrations
Nucleus (200 µm³):

Abundant transcription factor	1 nM (100,000 copies/ nucleus)
Rare transcription factor	10 pM (1,000 copies/ nucleus)
Serum	50-100 mg/ ml

IV. Some useful Conversiontables

Molar conversions for protein

100 pmol	µg
10,000 Da protein	1

100,000 Da protein	10

Protein/ DNA conversions

1 kb of DNA encodes 333 amino acids ~/= 3.7×10^4 Da

Protein	DNA
10,000 Da	270 dp
30,000 Da	810 dp
100,000 Da	2,7 dp

Nucleic acid content of a typical human cell

DNA per cell	
Total RNA per cell	~ 6 pg
Proportion of total RNA in nucelus	~ 10-30 pg
DNA:RNA in nucleus	~ 14%
Human genome size (haploid)	~2:1
Coding sequences/ genomic DNA	3.3×10^9 bp
Number of genes	3%
Active genes	$0.5-1 \times 10^5$
mRNA molecules	1.5×10^4
Typical mRNA size	$2 \times 10^5 - 1 \times 10^6$
	1900 nt

RNA distribution in a typical mammalian cell

RNA species	Relative amount
rRNA (28S, 18S, 5S)	80-85%
tRNAs, snRNAs, low MW species	15-20%
mRNAs	1-5%

RNA content in various cells and tissues

Source		Total RNA	mRNA (µg)
Cell cultures (10^7 cells)		30-500	0.3-25
	NIH/3T3	120	3
	HeLa	150	3
	COS-7	350	5
Mouse-developmental stages (per organism)	Unfertilized egg	0.43 ng	nd
	Oocyte	0.35 ng	nd

	2-cell	0.24 ng	nd
	8-16-cell	0.69 ng	nd
	32-cell	1.47 ng	nd
	13-day-old-embryo	450	13
Mouse tissue (100 mg)			
	Brain	120	5
	Heart	120	6
	Intestine	150	2
	Kidney	350	9
	Liver	400	14
	Lung	130	6
	Spleen	350	7

nd = not determined

Human blood*: cell, DNA, RNA, and protein content

	Leukocyes	Thrombocytes	Erythrocytes
Function	Immune response	Wound closing	O_2 & CO_2 transport
Cells per ml	$4-7 \times 10^6$	$3-4 \times 10^8$	5×10^9
DNA content	30-60 µg/ ml blood (6 pg/cell)		
RNa content	1-5 µg/ ml blood		
Hemoglobin content			~150 mg/ ml blood (30 pg/cell)
Plasma protein content		60-80 mg/ ml	

*From a healthy individual. The leukocyte concentration can vary from 2 x 106 per ml in cases of immunosuppression, to 40 x 10^{6} during inflammation, to 500 x 10^{6} during leukemia. The DNA and RNA content will vary accordingly.

zum Hauptmenü

EVIDENCE APPENDIX

ITEM NO. 13

Molecular Biology of the Cell, 4th Ed., Chapter 17, cited by
the Examiner in the October 2, 2008 Office Action
issued in co-pending application Serial No. 09/836,750

(also attached hereto as Exhibit A)

ALBERTS
JOHNSON
LEWIS
RAFF
ROBERTS
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Molecular Biology of the Cell

Bruce Alberts
Alexander Johnson
Julian Lewis
Martin Raff
Keith Roberts
Peter Walter

Molecular Biology of the Cell is the classic in-depth text reference in cell biology. By extracting fundamental concepts and meaning from this enormous and ever-growing field, the authors tell the story of cell biology, and create a coherent framework through which non-expert readers may approach the subject. Written in clear and concise language, and illustrated with original drawings, the book is enjoyable to read, and provides a sense of the excitement of modern biology. Molecular Biology of the Cell not only sets forth the current understanding of cell biology (updated as of Fall 2001), but also explores the intriguing implications and possibilities of that which remains unknown.

The hallmark features of previous editions continue in the Fourth Edition. The book is designed with a clean and open, single-column layout. The art program maintains a completely consistent format and style, and includes over 1,600 photographs, electron micrographs, and original drawings by the authors. Clear and concise concept headings introduce each section. Every chapter contains extensive references. Most important, every chapter had been subjected to a rigorous, collaborative revision process where, in addition to incorporating comments from expert reviewers, each co-author reads and reviews the other authors' prose. The result is a truly integrated work with a single authorial voice.

Bruce Alberts received his Ph.D. from Harvard University and is President of the National Academy of Sciences and Professor of

Biochemistry and Biophysics at the University of California, San Francisco. **Alexander Johnson** received his Ph.D. from Harvard University and is a Professor of Microbiology and Immunology at the University of California, San Francisco. **Julian Lewis** received his D.Phil. from the University of Oxford and is a Principal Scientist at the Imperial Cancer Research Fund, London. **Martin Raff** received his M.D. from McGill University and is at the Medical Research Council Laboratory for Molecular Cell Biology and Cell Biology Unit and in the Biology Department at University College London. **Keith Roberts** received his Ph.D. from the University of Cambridge and is Associate Research Director at the John Innes Centre, Norwich. **Peter Walter** received his Ph.D. from The Rockefeller University in New York and is Professor and Chairman of the Department of Biochemistry and Biophysics at the University of California, San Francisco, and an Investigator of the Howard Hughes Medical Institute.

Cell Biology Interactive
Artistic and Scientific Direction: Peter Walter
Narrated by: Julie Theriot
Production, Design, and Development: Mike Morales

Front cover Human Genome: Reprinted by permission from *Nature*, International Human Genome Sequencing Consortium, 409:860-921, 2001 © Macmillan Magazines Ltd. Adapted from an image by Francis Collins, NHGRI; Jim Kent, UCSC; Ewan Birney, EBI; and Darryl Leja, NHGRI; showing a portion of Chromosome 1 from the initial sequencing of the human genome.

Back cover In 1967, the British artist Peter Blake created a design classic. Nearly 35 years later Nigel Orme (illustrator), Richard Denyer (photographer), and the authors have together produced an affectionate tribute to Mr Blake's image. With its gallery of icons and

influences, its assembly created almost as much
complexity, intrigue and mystery as the original.
Drosophila, Arabidopsis, Dolly and the assembled
company tempt you to dip inside where, as in the
original, "a splendid time is guaranteed for all."
(Gunter Blobel, courtesy of The Rockefeller University; Marie
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EVIDENCE APPENDIX

ITEM NO. 14

**Isner U.S. Patent No. 5,980,887
cited by Appellant as Exhibit A in the Response
filed November 28, 2007**

[54] METHODS FOR ENHANCING ANGIOGENESIS WITH ENDOTHELIAL PROGENITOR CELLS

[75] Inventors: Jeffrey M. Isner, Weston; Takayuki Asahara, Arlington, both of Mass.

[73] Assignee: St. Elizabeth's Medical Center of Boston, Boston, Mass.

[21] Appl. No.: 08/744,882

[22] Filed: Nov. 8, 1996

[51] Int. Cl.⁶ A61K 35/12; A61K 48/00; A61K 38/18; A61K 38/19

[52] U.S. Cl. 424/93.7; 424/85.1; 424/85.2; 514/8; 514/44

[58] Field of Search 424/93.7, 85.4, 424/85.2; 435/325, 375; 514/2, 8, 44; 530/351; 53/23.5

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Primary Examiner—Christina Y. Chan
Assistant Examiner—Phillip Gambel
Attorney, Agent, or Firm—David G. Conlin; David S. Resnick; Dike, Bronstein, Roberts & Cushman, LLP

[57]   ABSTRACT

In accordance with the present invention, EC progenitors can be used in a method for regulating angiogenesis, i.e., enhancing or inhibiting blood vessel formation, in a selected patient and in some preferred embodiments for targeting specific locations. For example, the EC progenitors can be used to enhance angiogenesis or to deliver an angiogenesis modulator, e.g. anti- or pro-angiogenic agents, respectively to sites of pathologic or utilitarian angiogenesis. Additionally, in another embodiment, EC progenitors can be used to induce reendothelialization of an injured blood vessel, and thus reduce restenosis by indirectly inhibiting smooth muscle cell proliferation.

11 Claims, 7 Drawing Sheets

FIG. 1A

FIG. 1B

FIG. 1C
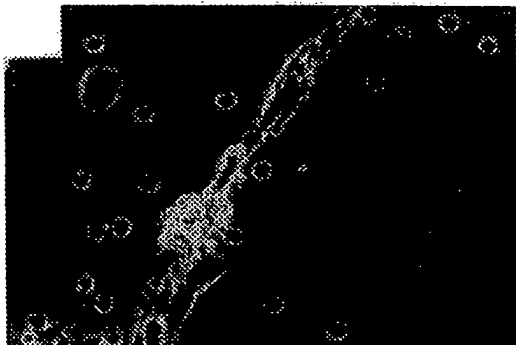
FIG. 1D

FIG. 1E

FIG. 1F

FIG. 1G

FIG. 2

CD34+/non
CD34+/COL
CD34+/FN
CD34-/FN

□ 12 HOURS
■ 2 DAYS

0   20   40   60   80   100
NUMBER OF ATTACHING CELL (/mm)

FIG. 5

0.1μM
1μM
10μM

Ach

1ng/ml
10ng/ml
100ng/ml

VEGF

0   5   10   15
NO (PICOMOLES/10^5 CELLS)

FIG. 7

CONTROL
VEGF
TNFα-prep
TNFα-prep/VEGF

□ 12 HOURS
▨ 3 DAYS
■ 7 DAYS

0   10   20   30
AT^{CD34+}CELLS/mm^2
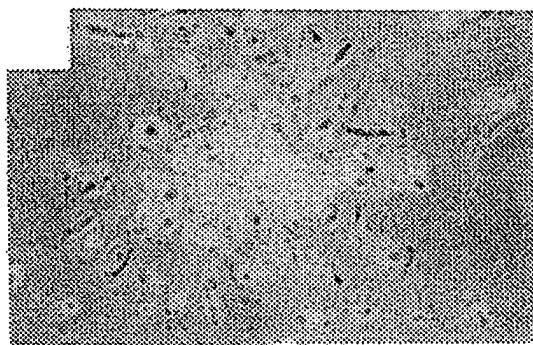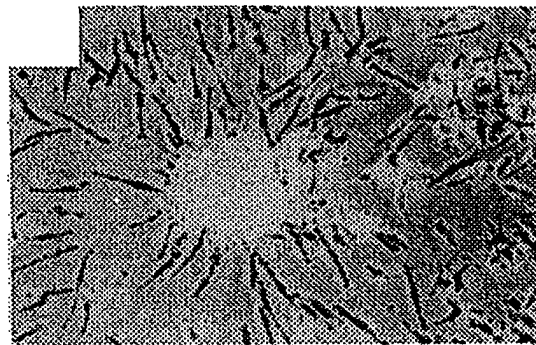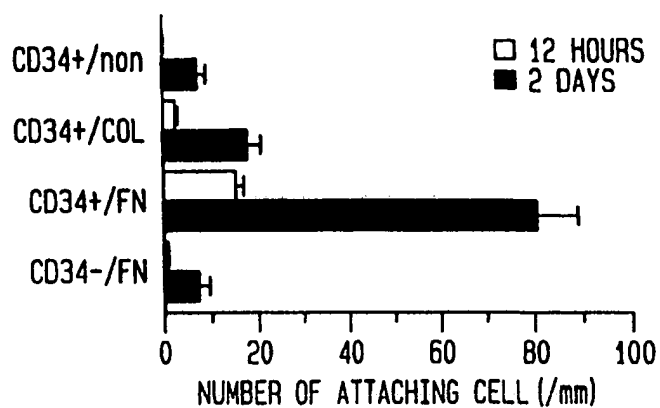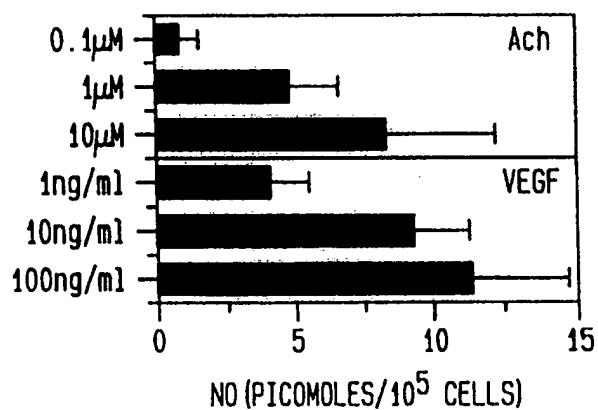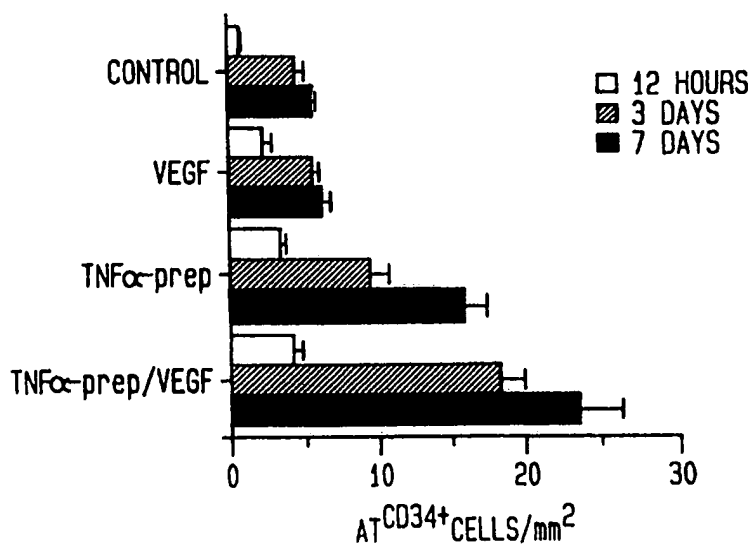
FIG. 3
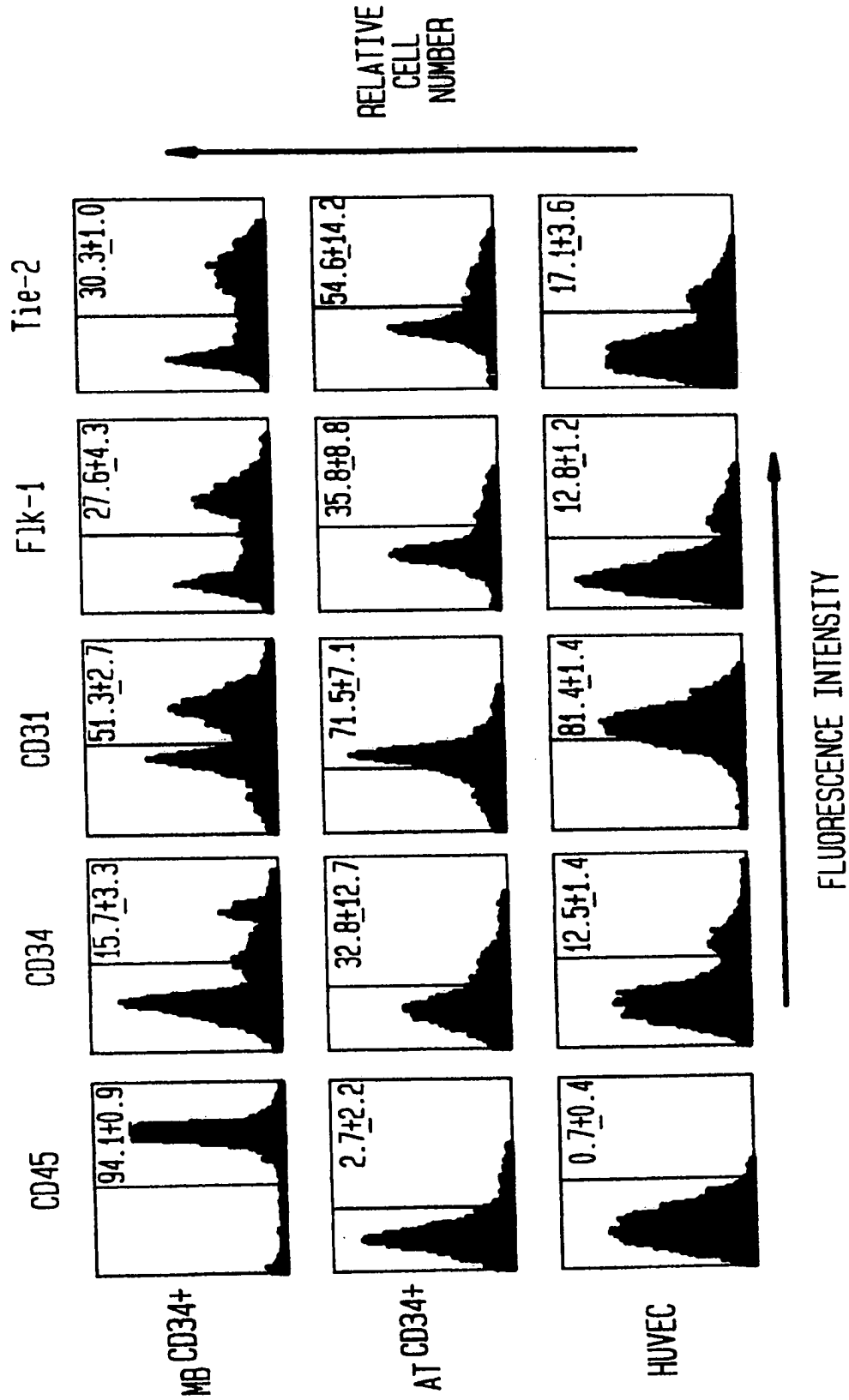
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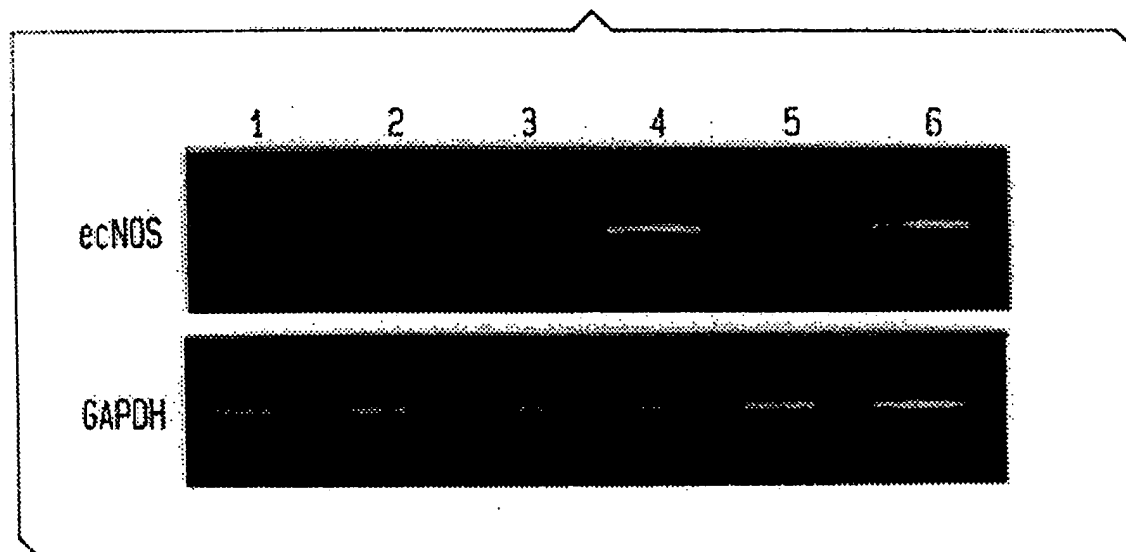
FIG. 9
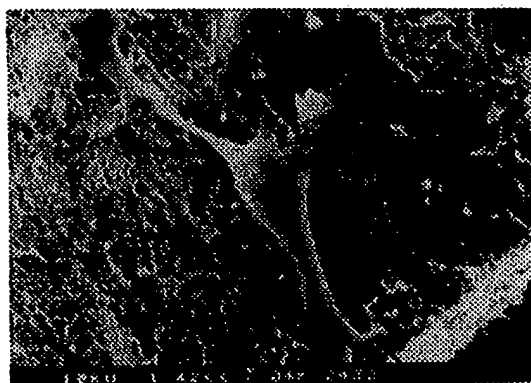
FIG. 6A
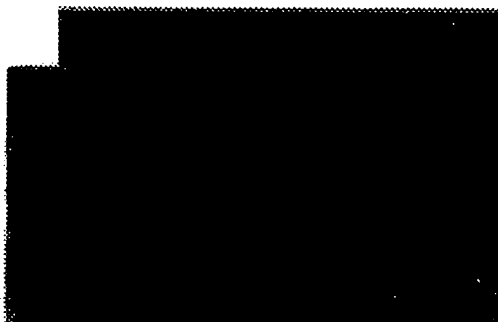
FIG. 6B

FIG. 6C
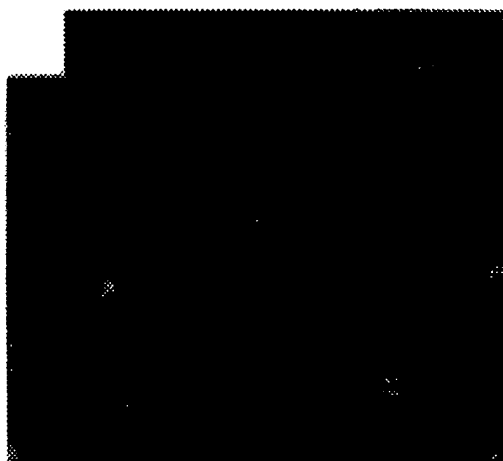
FIG. 6D
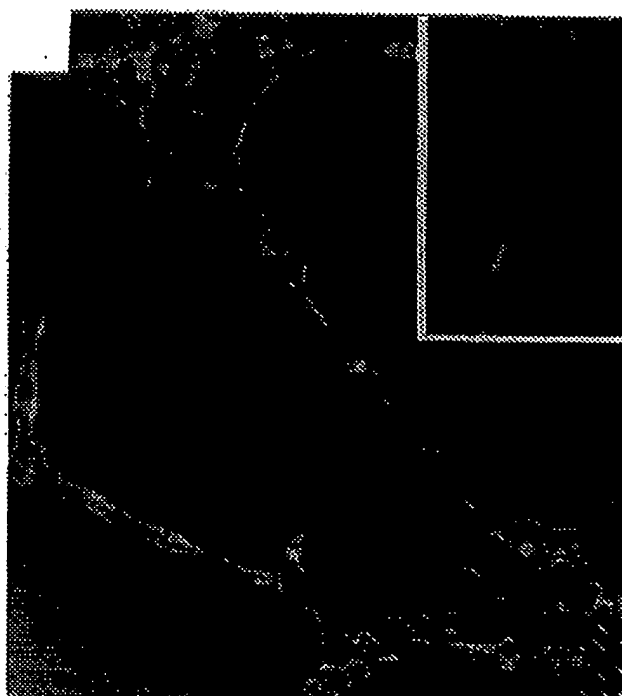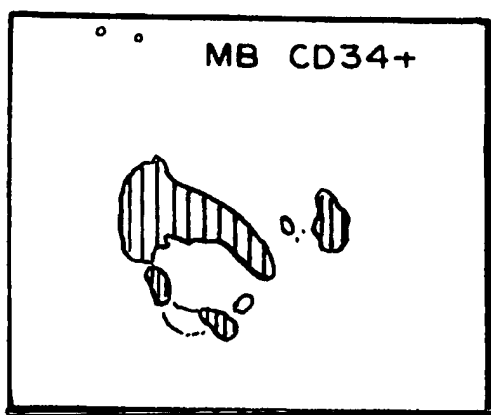
FIG.8A
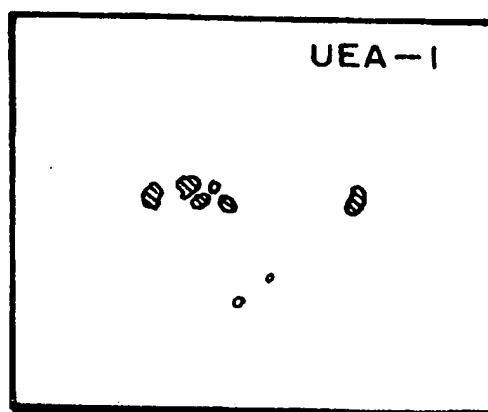
FIG.8B
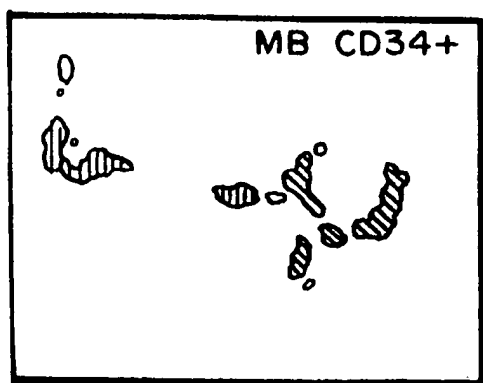
FIG.8C
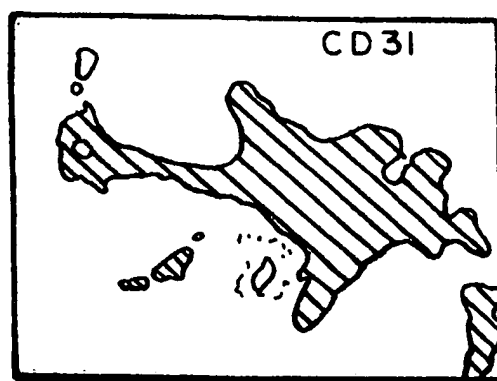
FIG.8D
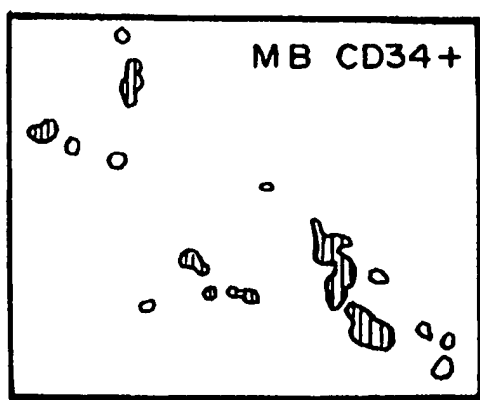
FIG.8E
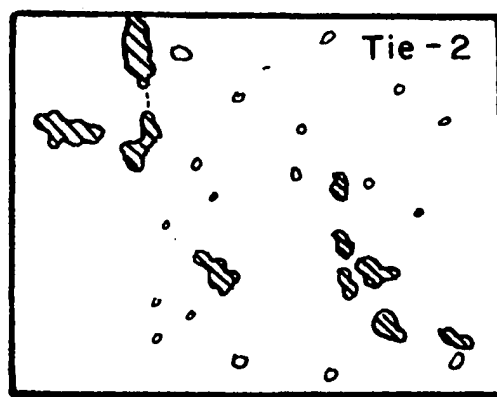
FIG.8F
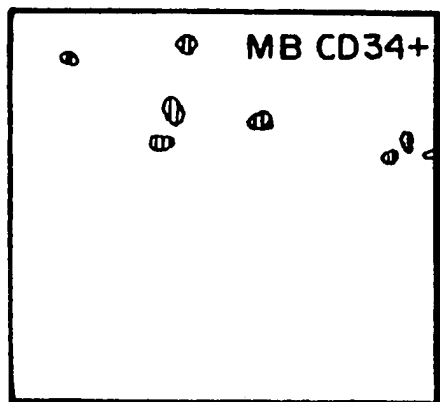
FIG.8G
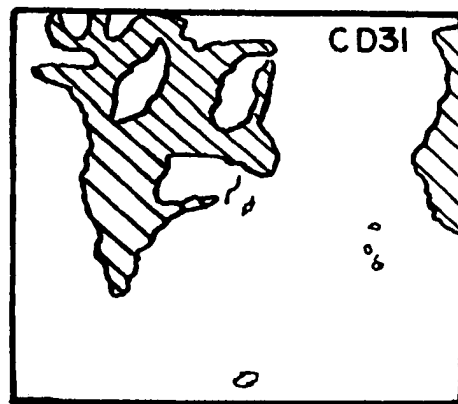
FIG.8H
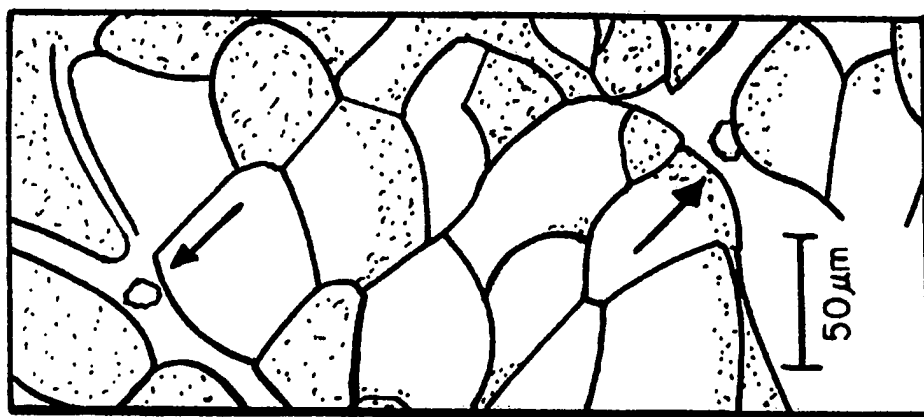
FIG.8I

FIG.8J

FIG.8K

1
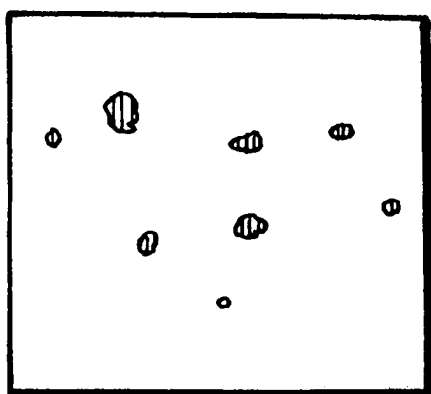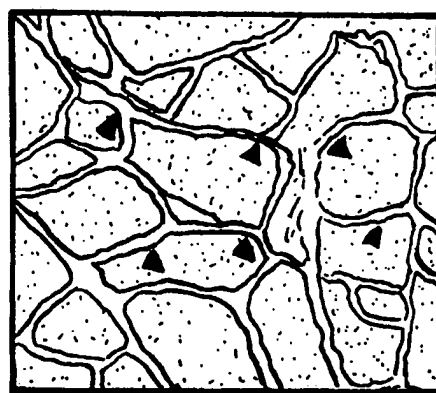
METHODS FOR ENHANCING ANGIOGENESIS WITH ENDOTHELIAL PROGENITOR CELLS

BACKGROUND OF THE INVENTION

Blood vessels are the means by which oxygen and nutrients are supplied to living tissues and waste products removed from living tissue. Angiogenesis is the process by which new blood vessels are formed, as reviewed, for example, by Folkman and Shing, J. Biol. Chem. 267 (16), 10931–10934 (1992). Thus angiogenesis is a critical process. It is essential in reproduction, development and wound repair. However, inappropriate angiogenesis can have severe consequences. For example, it is only after many solid tumors are vascularized as a result of angiogenesis that the tumors begin to grow rapidly and metastasize. Because angiogenesis is so critical to these functions, it must be carefully regulated in order to maintain health. The angiogenesis process is believed to begin with the degradation of the basement membrane by proteases secreted from endothelial cells (EC) activated by mitogens such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). The cells migrate and proliferate, leading to the formation of solid endothelial cell sprouts into the stromal space, then, vascular loops are formed and capillary tubes develop with formation of tight junctions and deposition of new basement membrane.

In the adults, the proliferation rate of endothelial cells is typically low compared to other cell types in the body. The turnover time of these cells can exceed one thousand days. Physiological exceptions in which angiogenesis results in rapid proliferation occurs under tight regulation are found in the female reproduction system and during wound healing.

The rate of angiogenesis involves a change in the local equilibrium between positive and negative regulators of the growth of microvessels. Abnormal angiogenesis occurs when the body loses its control of angiogenesis, resulting in either excessive or insufficient blood vessel growth. For instance, conditions such as ulcers, strokes, and heart attacks may result from the absence of angiogenesis normally required for natural healing. On the contrary, excessive blood vessel proliferation may favor tumor growth and spreading, blindness, psoriasis and rheumatoid arthritis.

The therapeutic implications of angiogenic growth factors were first described by Folkman and colleagues over two decades ago (Folkman, N. Engl. J. Med., 285:1182–1186 (1971)). Thus, there are instances where a greater degree of angiogenesis is desirable—wound and ulcer healing. Recent investigations have established the feasibility of using recombinant angiogenic growth factors, such as fibroblast growth factor (FGF) family (Yanagisawa-Miwa, et al., Science, 257:1401–1403 (1992) and Baffour, et al., J Vasc Surg, 16:181–91 (1992)), endothelial cell growth factor (ECGF)(Pu, et al., J Surg Res, 54:575–83 (1993)), and more recently, vascular endothelial growth factor (VEGF) to expedite and/or augment collateral artery development in animal models of myocardial and hindlimb ischemia (Takeshita, et al., Circulation, 90:228–234 (1994) and Takeshita, et al., J Clin Invest, 93:662–70 (1994)).

Conversely, there are also instances, where inhibition of angiogenesis is desirable. For example, many diseases are driven by persistent unregulated angiogenesis. In arthritis, new capillary blood vessels invade the joint and destroy cartilage. In diabetes, new capillaries invade the vitreous, bleed, and cause blindness. Ocular neovascularization is the most common cause of blindness. Tumor growth and

2

metastasis are angiogenesis-dependent. A tumor must continuously stimulate the growth of new capillary blood vessels for the tumor itself to grow.

The current treatment of these diseases is inadequate. Agents which prevent continued angiogenesis, e.g, drugs (TNP-470), monoclonal antibodies and antisense nucleic acids, are currently being tested. However, new agents that inhibit angiogenesis are need.

Recently, the feasibility of gene therapy for modulating angiogenesis has been demonstrated. For example, promoting angiogenesis in the treatment of ischemia was demonstrated in a rabbit model and in human clinical trials with VEGF using a Hydrogel-coated angioplasty balloon as the gene delivery system. Successful transfer and sustained expression of the VEGF gene in the vessel wall subsequently augmented neovascularization in the ischemic limb (Takeshita, et al., Laboratory Investigation, 75:487–502 (1996); Isner, et al., Lancet, 348:370 (1996)). In addition, it has been demonstrated that direct intramuscular injection of DNA encoding VEGF into ischemic tissue induces angiogenesis, providing the ischemic tissue with increased blood vessels (U.S. Ser. No. 08/545,998; Tsurumi et al., Circulation, In Press).

Alternative methods for regulating angiogenesis are still desirable for a number of reasons. For example, it is believed that native endothelial cell (EC) number and/or viability decreases over time. Thus, in certain patient populations, e.g., the elderly, the resident population of ECs that is competent to respond to administered angiogenic cytokines may be limited.

Moreover, while agents promoting or inhibiting angiogenesis may be useful at one location, they may be undesirable at another location. Thus, means to more precisely regulate angiogenesis at a given location are desirable.

SUMMARY OF THE INVENTION

We have now discovered that by using techniques similar to those employed for HSCs, EC progenitors can be isolated from circulating blood. In vitro, these cells differentiate into ECs. Indeed, one can use a multipotentiate undifferentiated cell as long as it is still capable of becoming an EC, if one adds appropriate agents to result in it differentiating into an EC.

We have also discovered that in vivo, heterologous, homologous, and autologous EC progenitor grafts incorporate into sites of active angiogenesis or blood vessel injury, i.e. they selectively migrate to such locations. This observation was surprising. Accordingly, one can target such sites by the present invention.

The present invention provides a method for regulating angiogenesis in a selected patient in need of a change in the rate of angiogenesis at a selected site. The change in angiogenesis necessary may be reduction or enhancement of angiogenesis. This is determined by the disorder to be treated. In accordance with the method of the present invention, an effective amount of an endothelial progenitor cell or modified version thereof to accomplish the desired result is administered to the patient.

In order to reduce undesired angiogenesis, for example, in the treatment of diseases such as rheumatoid arthritis, psoriasis, ocular neovascularization, diabetic retinopathy, neovascular glaucoma, angiogenesis-dependent tumors and tumor metastasis, a modified endothelial cell, having been modified to contain a compound that inhibits angiogenesis, e.g., a cytotoxic compound or angiogenesis inhibitor, can be administered.

To enhance angiogenesis, for example in the treatment of cerebrovascular ischemia, renal ischemia, pulmonary ischemia, limb ischemia, ischemic cardiomyopathy and myocardial ischemia, endothelial progenitor cells are administered. To further enhance angiogenesis an endothelial progenitor cell modified to express an endothelial cell mitogen may be used. Additionally, an endothelial cell mitogen or a nucleic acid encoding an endothelial cell mitogen can further be administered.

In another embodiment, the present invention provides methods of enhancing angiogenesis or treating an injured blood vessel. In accordance with these methods, endothelial progenitor cells are isolated from the patient, preferably from peripheral blood, and readministering to the patient. The patient may also be treated with endothelial cell mitogens to endothelial cell growth. The vessel injury can be the result of balloon angioplasty, deployment of an endovascular stent or a vascular graft.

The present invention also provides a method of screening for the presence of ischemic tissue or vascular injury in a patient. The method involves contacting the patient with a labelled EC progenitor and detecting the labelled cells at the site of the ischemic tissue or vascular injury.

The present invention also includes pharmaceutical products and kit for all the uses contemplated in the methods described herein.

Other aspects of the invention are disclosed infra.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A–1G show cell shape and formation. FIG. 1A shows spindle shaped attaching cells (AT^{CD34+}) 7 days after plating MB^{CD34+} on fibronectin with standard medium (10). Network formation (1B) and cord-like structures (1C) were observed 48 h after plating co-culture of MB^{CD34+}, labeled with Dil dye (Molecular Probe), and unlabeled MB^{CD34-} (ratio of 1:100) on fibronectin-coated dish. These cords consisted principally of Dil-labeled MB^{CD34+} derived cells (AT^{CD34+}). Beginning 12 h after co-culture, MB^{CD34+} derived cells demonstrated multiple foci of cluster formation (1D,1E,). AT^{CD34+} sprout from the periphery, while round cells remain in the center and detach from the cluster several days later. After 5 d, uptake of acLDL-Dil (Molecular Probe) was seen in AT^{CD34+} at the periphery but not the center of the cluster (1F,1G).

FIG. 2 shows the number of AT^{CD34+} 12 h and 3 d after single culture of MB^{CD34+} on plastic alone (CD34+/non), collagen coating (CD34+/COL), or fibronectin (CD34+/FN), and MB^{CD34-} on fibronectin (CD34−/FN). AT^{CD34+} yielded significantly higher number of cells at 12 h and 3 d when plated on fibronectin (p<0.05, by ANOVA).

FIG. 3 shows FACS analysis of freshly isolated MB^{CD34+}, AT^{CD34+} after 7 days in culture, and HUVECs. Cells were labeled with FITC using antibodies against CD34, CD31 (Biodesign); Flk-1, Tie-2 (Santa Cruz Biotechnology); and CD45. All results were confirmed by triplicate experiments. Shaded area of each box denotes negative antigen gate, white area denotes positive gate. Numbers indicated for individual gates denote percentage of cells determined by comparison with corresponding negative control labeling.

FIG. 4 shows expression of ecNOS mRNA in MB^{CD34-}, MB^{CD34+}, AT^{CD34+}, human coronary smooth muscle cells (HCSMCs) and HUVECs. DNA was reverse transcribed from $\approx 1 \times 10^6$ cells each. Equal aliquots of the resulting DNA were amplified by PCR (40 cycles) with paired primers (sense/antisense: AAG ACA TTT TCG GGC TCA CGC TGC GCA CCC/TGG GGT AGG CAC TTT AGT AGT TCT

CCT AAC, SEQ ID NO:2) to detect ecNOS mRNA. Equal aliquots of the amplified product were analyzed on a 1% agarose gel. Only a single band was observed, corresponding to the expected size (548 bp) for ecNOS. Lane 1=MB^{CD34-}, Lane 2=MB^{CD34+}, Lane 3=AT^{CD34+} after 3 d, Lane 4=AT^{CD34+} after 7 d, Lane 5=HCSMCs, Lane 6=HUVECs.

FIG. 5 is a graph illustrating NO release from AT^{CD34+} was measured with an NO-specific polarographic electrode connected to an NO meter (Iso-NO, World Precision Instruments) (17). Calibration of NO electrode was performed daily before experimental protocol according to the following equation: $2KNO_2+2KI+2H_2SO_4 \rightarrow 2NO+I_2+2H_2O+2K_2SO_4$. standard calibration curve was obtained by adding graded concentrations of KNO_2 (0–500 nmol/L) to calibration solution containing KI and H_2SO_4. Specificity of the electrode to NO was previously documented by measurement of NO from authentic NO gas (18). AT^{CD34+} cultured in 6-well plate were washed and then bathed in 5 ml of filtered Krebs-Henseleit solution. Cell plates were kept on a slide warmer (Lab Line Instruments) to maintain temperature between 35 and 37° C. For NO measurement, sensor probe was inserted vertically into the wells, and the tip of the electrode remained 2 mm under the surface of the solution. Measurement of NO, expressed as pmol/10^5 cells, was performed in a well with incremental doses of VEGF (1, 10, 100 ng/ml) and Ach (0.1, 1, 10 μM). HUVECs and bovine aortic ECs were employed as positive controls. For negative control, HCSMCs, NO was not detectable. All values reported represent means of 10 measurements for each group.

FIGS. 6A–6D show co-culture of MB^{CD34+} with HUVECs. Freshly isolated MB^{CD34+} were labeled with Dil dye and plated on a confluent HUVEC monolayer attached to a fibronectin-coated chamber slide at a density of 278 cells/mm^2 (Nunc). Differentiation of MB^{CD34+} into spindle shaped attaching cells (AT^{CD34+}) (red fluorescence) was observed among HUVECs within 12 h (6A). The AT^{CD34+} number increased on monolayer for 3 d (6B), while meshwork structures were observed in some areas (6C). Three days after co-culture, both cells were re-seeded on Matrigel (Becton Dickinson)-coated slides and within 12 h disclosed capillary network formation, consisting of Dil-labeled AT^{CD34+} and HUVECs (6D).

FIG. 7 shows the effect of activated ECs and VEGF on MB^{CD34+} differentiation was investigated by pretreatment of HUVEC with TNF-α (20 ng/ml) for 12 h, and/or incubation of AT^{CD34+}/HUVEC co-culture with VEGF (50 ng/ml).

FIGS. 8A–8K show sections retrieved from ischemic hindlimb following in vivo administration of heterologous (FIGS. 8A–8H), homologous (8I), and autologous (8J,8K) EC progenitors. (8A,8B) Red fluorescence in small intermuscular artery 6 wks after injection of Dil-labeled MB^{CD34+}. Green fluorescence denotes EC-specific lectin UEA-1. (8C) Dil (red) and CD31 (green) in capillaries between muscles, photographed through double filter 4 wks after Dil-labeled MB^{CD34+} injection. (8D) Same capillary structure as in (C), showing CD31 expression by MB^{CD34+} which have been incorporated into host capillary structures expressing CD31. (8E,8F) Immunostaining 2 wks after MB^{CD34+} injection shows capillaries comprised of Dil-labeled MB^{CD34+} derived cells expressing tie-2 receptor (green fluorescence). Most MB^{CD34+} derived cells are tie-2 positive, and are integrated with some tie-2 positive native (host) capillary cells identified by absence of red fluorescence. (8G,8H) Two wks after injection of Dil-labeled MB^{CD34-}. Although isolated MB^{CD34-} derived cells (red) can be observed between muscles, but these cells do not express CD31.

(8I) Immunohistochemical, β-galactosidase staining of muscle harvested from ischemic limb of B6, 129 mice 4 wks following administration of MB^{Flk-1+} isolated from β-galactosidase transgenic mice. Cells overexpressing β-galactosidase (arrows) have been incorporated into capillaries and small arteries; these cells were identified as ECs by anti-CD31 antibody and BS-1 lectin.

(8J,8K) Sections of muscles harvested from rabbit ischemic hindlimb 4 wks after administration of autologous MB^{CD34+}. DiI fluorescence (J) indicates localization of MB^{CD34+} derived cells in capillaries seen in phase contrast photomicrograph (8K). Each scale bar indicates 50 μm.

FIG. 9 is a photograph from a scanning electron microscope showing that EC progenitors had adhered to the denuded arterial surface and assumed a morphology suggestive of endothelial cells.

DETAILED DESCRIPTION OF THE INVENTION

We have now discovered a means to regulate angiogenesis, to promote angiogenesis in certain subject populations, and to more precisely target certain tissues. These methods all involve the use of endothelial cell progenitors. One preferred progenitor cell is an angioblast.

Post-natal neovascularization is believed to result exclusively from the proliferation, migration, and remodeling of fully differentiated endothelial cells (ECs) derived from pre-existing native blood vessels (1). This adult paradigm, referred to as angiogenesis, contrasts with vasculogenesis, the term applied to formation of embryonic blood vessels from EC progenitors (2).

In contrast to angiogenesis, vasculogenesis typically begins as a cluster formation, or blood island, comprised of EC progenitors (e.g. angioblasts) at the periphery and hematopoietic stem cells (HSCs) at the center (3). In addition to this intimate and predictable spatial association, such EC progenitors and HSCs share certain common antigenic determinants, including flk-1, tie-2, and CD-34. Consequently, these progenitor cells have been interpreted to derive from a common hypothetical precursor, the hemangioblast (3,4).

The demonstration that transplants of HSCs derived from peripheral blood can provide sustained hematopoietic recovery constitutes inferential evidence for circulating stem cells. (5). This observation is now being exploited clinically as an alternative to bone marrow transplantation.

We have now discovered that by using techniques similar to those employed for HSCs, EC progenitors can be isolated from circulating blood. In vitro, these cells differentiate into ECs. Indeed, one can use a multipotentiate undifferentiated cell as long as it is still capable of becoming an EC, if one adds appropriate agents to result in it differentiating into an EC.

We have also discovered that in vivo, heterologous, homologous, and autologous EC progenitor grafts incorporate into sites of active angiogenesis or blood vessel injury, i.e. they selectively migrate to such locations. This observation was surprising. Accordingly, one can target such sites by the present invention.

In accordance with the present invention, EC progenitors can be used in a method for regulating angiogenesis, i.e., enhancing or inhibiting blood vessel formation, in a selected patient and in some preferred embodiments for targetting specific locations. For example, the EC progenitors can be used to enhance angiogenesis or to deliver an angiogenesis

modulator, e.g. anti- or pro-angiogenic agents, respectively to sites of pathologic or utilitarian angiogenesis. Additionally, in another embodiment, EC progenitors can be used to induce reendothelialization of an injured blood vessel, and thus reduce restenosis by indirectly inhibiting smooth muscle cell proliferation.

In one preferred embodiment the EC cells can be used alone to potentiate a patient for angiogenesis. Some patient population, typically elderly patients, may have either a limited number of ECs or a limited number of functional ECs. Thus, if one desires to promote angiogenesis, for example, to stimulate vascularization by using a potent angiogenesis promotor such as VEGF, such vascularization can be limited by the lack of ECs. However, by administering the EC progenitors one can potentiate the vascularization in those patients.

Accordingly, the present method permits a wide range of strategies designed to modulate angiogenesis such as promoting neovascularization of ischemic tissues (24). EC mitogens such as VEGF and bFGF, for example, have been employed to stimulate native ECs to proliferate, migrate, remodel and thereby form new sprouts from parent vessels (25). A potentially limiting factor in such therapeutic paradigms is the resident population of ECs that is competent to respond to administered angiogenic cytokines. The finding that NO production declines as a function of age (26) may indicate a reduction in EC number and/or viability that could be addressed by autologous EC grafting. The success demonstrated to date with autologous grafts of HSCs derived from peripheral blood (5) supports the clinical feasibility of a "supply side" approach to therapeutic angiogenesis. The in vivo data set forth herein indicate that autologous EC transplants are feasible, and the in vitro experiments indicate that EC progenitors (MB^{CD34+} derived ECs) can be easily manipulated and expanded ex vivo.

Our discovery that these EC progenitors home to foci of angiogenesis makes these cells useful as autologous vectors for gene therapy and diagnosis of ischemia or vacular injury. For example, these cells can be utilized to inhibit as well as augment angiogenesis. For anti-neoplastic therapies, for example, EC progenitors can be transfected with or coupled to cytotoxic agents, cytokines or co- stimulatory molecules to stimulate an immune reaction, other anti-tumor drugs or angiogenesis inhibitors. For treatment of regional ischemia, angiogenesis could be amplified by prior transfection of EC progenitors to achieve constitutive expression of angiogenic cytokines and/or selected matrix proteins (27). In addition, the EC progenitors may be labelled, e.g., radiolabelled, administered to a patient and used in the detection of ischemic tissue or vacular injury.

EC progenitors may be obtained from human mononuclear cells obtained from peripheral blood or bone marrow of the patient before treatment. EC progenitors may also be obtained from heterologous or autologous umbilical cord blood. Peripheral blood is preferred due to convenience. The leukocyte fraction of peripheral blood is most preferred. EC progenitors may be isolated using antibodies that recognize EC progenitor specific antigens on immature human hematopoietic progenitor cells (HSCs). For example, CD34 is commonly shared by EC progenitor and HSCs. CD34 is expressed by all HSCs but is lost by hematopoietic cells as they differentiate (6). It is also expressed by many, including most activated, ECs in the adult (7). Flk-1, a receptor for vascular endothelial growth factor (VEGF) (8), is also expressed by both early HSCs and ECs, but ceases to be expressed in the course of hematopoietic differentiation (9).

To obtain the EC progenitors from peripheral blood about 5 ml to about 500 ml of blood is taken from the patient. Ppreferably, about 50 ml to about 200 ml of blood is taken.

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EC progenitors can be expanded in vivo by administration of recruitment growth factors, e.g., GM-CSF and IL-3, to the patient prior to removing the progenitor cells.

Methods for obtaining and using hematopoietic progenitor cells in autologous transplantation are disclosed in U.S. Pat. No. 5,199,942, the disclosure of which is incorporated by reference.

Once the progenitor cells are obtained by a particular separation technique, they may be administered to a selected patient to treat a number of conditions including, for example, unregulated angiogenesis or blood vessel injury. The cells may also be stored in cryogenic conditions. Optionally, the cells may be expanded ex vivo using, for example, the method disclosed by U.S. Pat. No. 5,541,103, the disclosure of which is incorporated by reference.

The progenitor cells are administered to the patient by any suitable means, including, for example, intravenous infusion, bolus injection, and site directed delivery via a catheter. Preferably, the progenitor cells obtained from the patient are readministered. Generally, from about 10^6 to about 10^{18} progenitor cells are administered to the patient for transplantation.

Depending on the use of the progenitor cells, various genetic material may be delivered to the cell. The genetic material that is delivered to the EC progenitors may be genes, for example, those that encode a variety of proteins including anticancer agents. Such genes include those encoding various hormones, growth factors, enzymes, cytokines, receptors, MHC molecules and the like. The term "genes" includes nucleic acid sequences both exogenous and endogenous to cells into which a virus vector, for example, a pox virus such as swine pox containing the human TNF gene may be introduced. Additionally, it is of interest to use genes encoding polypeptides for secretion from the EC progenitors so as to provide for a systemic effect by the protein encoded by the gene. Specific genes of interest include those encoding TNF, TGF-α, TGF-β, hemoglobin, interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-5, interleukin-6, interleukin-7, interleukin-8, interleukin-9, interleukin-10, interleukin-11, interleukin-12 etc., GM-CSF, G-CSF, M-CSF, human growth factor, co-stimulatory factor B7, insulin, factor VIII, factor IX, PDGF, EGF, NGF, IL-ira, EPO, β-globin, EC mitogens and the like, as well as biologically active muteins of these proteins. The gene may further encode a product that regulates expression of another gene product or blocks one or more steps in a biological pathway. In addition, the gene may encode a toxin fused to a polypeptide, e.g., a receptor ligand, or an antibody that directs the toxin to a target, such as a tumor cell. Similarly, the gene may encode a therapeutic protein fused to a targeting polypeptide, to deliver a therapeutic effect to a diseased tissue or organ.

The cells can also be used to deliver genes to enhance the ability of the immune system to fight a particular disease or tumor. For example, the cells can be used to deliver one or more cytokines (e.g., IL-2) to boost the immune system and/or one or more antigens.

These cells may also be used to selectively administer drugs, such as an antiangiogenesis compound such as O-chloroacetyl carbamoyl fumagillol (TNP-470). Preferably the drug would be incorporated into the cell in a vehicle such as a liposome, a timed released capsule, etc. The EC progenitor would then selectively hone in on a site of active angiogenesis such as a rapidly growing tumor where the compound would be released. By this method, one can reduce undesired side effects at other locations.

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In one embodiment, the present invention may be used to enhance blood vessel formation in ischemic tissue, i.e., a tissue having a deficiency in blood as the result of an ischemic disease. Such tissues can include, for example, muscle, brain, kidney and lung. Ischemic diseases include, for example, cerebrovascular ischemia, renal ischemia, pulmonary ischemia, limb ischemia, ischemic cardiomyopathy and myocardial ischemia.

If it is desirable to further enhance angiogenesis, endothelial cell mitogens may also be administered to the patient in conjunction with, or subsequent to, the administration of the EC progenitor cells. Endothelial cell mitogens can be administered directly, e.g., intra-arterially, intramuscularly, or intravenously, or nucleic acid encoding the mitogen may be used. See, Baffour, et al., supra (bFGF); Pu, et al, *Circulation*, 88:208–215 (1993) (aFGF); Yanagisawa-Miwa, et al., supra (bFGF); Ferrara, et al., *Biochem. Biophys. Res. Commun.*, 161:851–855 (1989) (VEGF); (Takeshita, et al., *Circulation*, 90:228–234 (1994)).

The nucleic acid encoding the EC mitogen can be administered to a blood vessel perfusing the ischemic tissue or to a site of vascular injury via a catheter, for example, a hydrogel catheter, as described by U.S. Ser. No. 08/675,523, the disclosure of which is herein incorporated by reference. The nucleic acid also can be delivered by injection directly into the ischemic tissue using the method described in U.S. Ser. No. 08/545,998.

As used herein the term "endothelial cell mitogen" means any protein, polypeptide, mutein or portion that is capable of, directly or indirectly, inducing endothelial cell growth. Such proteins include, for example, acidic and basic fibroblast growth factors (aFGF and bFGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor α and β (TGF-α and TFG-β), platelet-derived endothelial growth factor (PD-ECGF), platelet-derived growth factor (PDGF), tumor necrosis factor a (TNF-α), hepatocyte growth factor (HGF), insulin like growth factor (IGF), erythropoietin, colony stimulating factor (CSF), macrophage-CSF (M-CSF), granulocyte/ macrophage CSF (GM-CSF) and nitric oxidesynthase (NOS). See, Klagsbrun, et al., *Annu. Rev. Physiol.*, 53:217–239 (1991); Folkman, et al., *J. Biol. Chem.*, 267:10931–10934 (1992) and Symes, et al., *Current Opinion in Lipidology*, 5:305–312 (1994). Muteins or fragments of a mitogen may be used as long as they induce or promote EC cell growth.

Preferably, the endothelial cell mitogen contains a secretory signal sequence that facilitates secretion of the protein. Proteins having native signal sequences, e.g., VEGF, are preferred. Proteins that do not have native signal sequences, e.g., bFGF, can be modified to contain such sequences using routine genetic manipulation techniques. See, Nabel et al., *Nature*, 362:844 (1993).

The nucleotide sequence of numerous endothelial cell mitogens, are readily available through a number of computer data bases, for example, GenBank, EMBL and Swiss-Prot. Using this information, a DNA segment encoding the desired may be chemically synthesized or, alternatively, such a DNA segment may be obtained using routine procedures in the art, e.g, PCR amplification. A DNA encoding VEGF is disclosed in U.S. Pat. No. 5,332,671, the disclosure of which is herein incorporated by reference.

In certain situations, it may be desirable to use nucleic acids encoding two or more different proteins in order optimize the therapeutic outcome. For example, DNA encoding two proteins, e.g., VEGF and bFGF, can be used,

and provides an improvement over the use of bFGF alone. Or an angiogenic factor can be combined with other genes or their encoded gene products to enhance the activity of targeted cells, while simultaneously inducing angiogenesis, including, for example, nitric oxide synthase, L-arginine, fibronectin, urokinase, plasminogen activator and heparin.

The term "effective amount" means a sufficient amount of compound, e.g. nucleic acid delivered to produce an adequate level of the endothelial cell mitogen, i.e., levels capable of inducing endothelial cell growth and/or inducing angiogenesis. Thus, the important aspect is the level of mitogen expressed. Accordingly, one can use multiple transcripts or one can have the gene under the control of a promoter that will result in high levels of expression. In an alternative embodiment, the gene would be under the control of a factor that results in extremely high levels of expression, e.g., tat and the corresponding tar element.

The EC progenitors may also be modified ex vivo such that the cells inhibit angiogenesis. This can be accomplished, for example, by introducing DNA encoding angiogenesis inhibiting agents to the cells, using for example the gene transfer techniques mentioned herein. Angiogenesis inhibiting agents include, for example, proteins such as thrombospondin (Dameron et al., Science 265:1582–1584 (1994)), angiostatin (O'Reilly et al., Cell 79:315–328 (1994), IFN-alpha (Folkman, J. Nature Med. 1:27–31 (1995)), transforming growth factor beta, tumor necrosis factor alpha, human platelet factor 4 (PF4); substances which suppress cell migration, such as proteinase inhibitors which inhibit proteases which may be necessary for penetration of the basement membrane, in particular, tissue inhibitors of metalloproteinase TIMP-1 and TIMP-2; and other proteins such as protamine which has demonstrated angiostatic properties, decoy receptors, drugs such as analogues of the angioinhibin fumagillin, e.g., TNP-470 (Ingber et al., Nature 348:555–557 (1990), antibodies or antisense nucleic acid against angiogenic cytokines such as VEGF. Alternatively, the cells may be coupled to such angiogenesis inhibiting agent.

If the angiogenesis is associated with neoplastic growth the EC progenitor cell may also be transfected with nucleic acid encoding, or coupled to, anti-tumor agents or agents that enhance the immune system. Such agents include, for example, TNF, cytokines such as interleukin (IL) (e.g., IL-2, IL-4, IL-10, IL-12), interferons (IFN) (e.g., IFN-γ) and co-stimulatory factor (e.g., B7). Preferably, one would use a multivalent vector to deliver, for example, both TNF and IL-2 simultaneously.

The nucleic acids are introduced into the EC progenitor by any method which will result in the uptake and expression of the nucleic acid by the cells. These can include vectors, liposomes, naked DNA, adjuvant-assisted DNA, catheters, gene gun, etc.

Vectors include chemical conjugates such as described in WO 93/04701, which has targeting moiety (e.g. a ligand to a cellular surface receptor), and a nucleic acid binding moiety (e.g. polylysine), viral vector (e.g. a DNA or RNA viral vector), fusion proteins such as described in PCT/US 95/02140 (WO 95/22618) which is a fusion protein containing a target moiety (e.g. an antibody specific for a target cell) and a nucleic acid binding moiety (e.g. a protamine), plasmids, phage, etc. The vectors can be chromosomal, non-chromosomal or synthetic.

Preferred vectors include viral vectors, fusion proteins and chemical conjugates. Retroviral vectors include moloney murine leukemia viruses and HIV-based viruses. One

preferred HIV-based viral vector comprises at least two vectors wherein the gag and pol genes are from an HIV genome and the env gene is from another virus. DNA viral vectors are preferred. These vectors include pox vectors such as orthopox or avipox vectors, herpesvirus vectors such as a herpes simplex I virus (HSV) vector [Geller, A. I. et al., J. Neurochem, 64: 487 (1995); Lim, F., et al., in DNA Cloning: Mammalian Systems, D. Glover, Ed. (Oxford Univ. Press, Oxford England) (1995); Geller, A. I. et al., Proc Natl. Acad. Sci.: U.S.A.:90 7603 (1993); Geller, A. I., et al., Proc Nat. Acad. Sci USA: 87:1149 (1990)], Adenovirus Vectors [LeGal LaSalle et al., Science, 259:988 (1993); Davidson, et al., Nat. Genet 3: 219 (1993); Yang, etal., J. Virol. 69: 2004 (1995)] and Adeno-associated Virus Vectors [Kaplitt, M. G., etal., Nat. Genet. 8:148 (1994)].

Pox viral vectors introduce the gene into the cells cytoplasm. Avipox virus vectors result in only a short term expression of the nucleic acid. Adenovirus vectors, adeno-associated virus vectors and herpes simplex virus (HSV) vectors are preferred for introducing the nucleic acid into neural cells. The adenovirus vector results in a shorter term expression (about 2 months) than adeno-associated virus (about 4 months), which in turn is shorter than HSV vectors. The particular vector chosen will depend upon the target cell and the condition being treated. The introduction can be by standard techniques, e.g. infection, transfection, transduction or transformation. Examples of modes of gene transfer include e.g., naked DNA, $CaPO_4$ precipitation, DEAE dextran, electroporation, protoplast fusion, lipofecton, cell microinjection, viral vectors and use of the "gene gun".

To simplify the manipulation and handling of the nucleic acid encoding the protein, the nucleic acid is preferably inserted into a cassette where it is operably linked to a promoter. The promoter must be capable of driving expression of the protein in cells of the desired target tissue. The selection of appropriate promoters can readily be accomplished. Preferably, one would use a high expression promoter. An example of a suitable promoter is the 763-base-pair cytomegalovirus (CMV) promoter. The Rous sarcoma virus (RSV) (Davis, et al., Hum Gene Ther 4:151 (1993)) and MMT promoters may also be used. Certain proteins can expressed using their native promoter. Other elements that can enhance expression can also be included such as an enhancer or a system that results in high levels of expression such as a tat gene and tar element. This cassette can then be inserted into a vector, e.g., a plasmid vector such as pUC118, pBR322, or other known plasmid vectors, that includes, for example, an E. coli origin of replication. See, Sambrook, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory press, (1989). The plasmid vector may also include a selectable marker such as the β-lactamase gene for ampicillin resistance, provided that the marker polypeptide does not adversely effect the metabolism of the organism being treated. The cassette can also be bound to a nucleic acid binding moiety in a synthetic delivery system, such as the system disclosed in WO 95/22618.

If desired, the preselected compound, e.g. a nucleic acid such as DNA may also be used with a microdelivery vehicle such as cationic liposomes and adenoviral vectors. For a review of the procedures for liposome preparation, targeting and delivery of contents, see Mannino and Gould-Fogerite, BioTechniques, 6:682 (1988). See also, Felgner and Holm, Bethesda Res. Lab. Focus, 11(2):21 (1989) and Maurer, R. A., Bethesda Res. Lab. Focus, 11(2):25 (1989).

Replication-defective recombinant adenoviral vectors, can be produced in accordance with known techniques. See, Quantin, et al., Proc. Natl. Acad. Sci. USA, 89:2581–2584

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(1992); Stratford-Perricadet, et al., *J. Clin. Invest.*, 90:626–630 (1992); and Rosenfeld, et al., *Cell*, 68:143–155 (1992).

The effective dose of the nucleic acid will be a function of the particular expressed protein, the target tissue, the patient and his or her clinical condition. Effective amount of DNA are between about 1 and 4000 μg, more preferably about 1000 and 2000, most preferably between about 2000 and 4000.

Alternatively, the EC progenitors may be used to inhibit angiogenesis and/or neoplastic growth by delivering to the site of angiogenesis a cytotoxic moiety coupled to the cell. The cytotoxic moiety may be a cytotoxic drug or an enzymatically active toxin of bacterial, fungal or plant origin, or an enzymatically active polypeptide chain or fragment ("A chain") of such a toxin. Enzymatically active toxins and fragments thereof are preferred and are exemplified by diphtheria toxin A fragment, non-binding active fragments of diphtheria toxin, exotoxin A (from Pseudomonas aeruginosa), ricin A chain, abrin A chain, modeccin A chain, alphasarcin, certain Aleurites fordii proteins, certain Dianthin proteins, Phytolacca americana proteins (PAP, PAPII and PAP-S), Momordica charantia inhibitor, curcin, crotin, Saponaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, and enomycin, Ricin A chain, Pseudomonas aeruginosa exotoxin A and PAP are preferred.

Conjugates of the EC progenitors and such cytotoxic moieties may be made using a variety of coupling agents. Examples of such reagents are N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters such as dimethyl adeipimidate HCl, active esters such as disuccinimidyl suberate, aldehydes such as glutaradehyde, bis-azido compounds such as bis(p-diazoniumbenzoyl)-ethylenediamine, diisocyanates such as tolylene 2,6-diisocyante, and bis-active fluorine compounds such as 1,5-difluoro-2,4-dinitrobenzene.

The enzymatically active polypeptide of the toxins may be recombinantly produced. Recombinantly produced ricin toxin A chain (rRTA) may be produced in accordance with the methods disclosed in PCT W085/03508 published Aug. 15, 1985. Recombinantly produced diphtheria toxin A chain and non-binding active fragments thereof are also described in PCT W085/03508 published Aug. 15, 1985.

The methods of the present invention may be used to treat blood vessel injuries that result in denuding of the endothelial lining of the vessel wall. For example, primary angioplasty is becoming widely used for the treatment of acute myocardial infarction. In addition, endovascular stents are becoming widely used as an adjunct to balloon angioplasty. Stents are useful for rescuing a sub-optimal primary result as well as for diminishing restenosis. To date, however, the liability of the endovascular prosthesis has been its susceptibility to thrombotic occlusion in approximately 3% of patients with arteries 3.3 mm or larger. If patients undergo stent deployment in arteries smaller than this the incidence of sub-acute thrombosis is even higher. Sub-acute thrombosis is currently prevented only by the aggressive use of anticoagulation. The combination of vascular intervention and intense anticoagulation creates significant risks with regard to peripheral vascular trauma at the time of the stent/angioplasty procedure. Acceleration of reendothelialization by adminstration of EC progenitors to a patient undergoing, or subsequent to, angioplasty and/or stent deployment can stabilize an unstable plaque and prevent re-occlusion.

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The method of the present invention may be used in conjunction with the method for the treatment of vascular injury disclosed in PCT/US96/15813.

In addition, the methods of the present invention may be used to accelerate the healing of graft tissue, e.g., vascular grafts.

The present invention also includes pharmaceutical products for all the uses contemplated in the methods described herein. For example, there is a pharmaceutical product, comprising nucleic acid encoding an endothelial cell mitogen and EC progenitors, in a physiologically acceptable administrable form.

The present invention further includes a kit for the in vivo systemic introduction of an EC progenitor and an endothelial cell mitogen or nucleic acid encoding the same into a patient. Such a kit includes a carrier solution, nucleic acid or mitogen, and a means of delivery, e.g., a catheter or syringe. The kit may also include instructions for the administration of the preparation.

All documents mentioned herein are incorporated by reference herein in their entirety.

The present invention is further illustrated by the following examples. These examples are provided to aid in the understanding of the invention and are not construed as a limitation thereof.

EXAMPLES

Method and Materials

Human peripheral blood was obtained using a 20 gauge intravenous catheter, discarding the first 3 ml. Leukocyte fraction of blood was obtained by Ficoll density gradient centrifugation and plated on plastic tissue culture for 1 hr to avoid contamination by differentiated adhesive cells.

Fluorescent activated cell sorting (FACS) was carried out with $>1{\times}10^6$ CD34 positive and negative mononuclear blood cells (MB^{CD34+}, MB^{CD34-}). Cells were analyzed with Becton-Dickinson FACS sorter and the lysis II analysis program using antibodies to CD34 (Biodesign).

M-199 medium with 20% FBS and bovine brain extract (Clonetics) was used as standard medium for all cell culture experiments.

C57BL/6Jx129/SV background male mice (Hirlan), 3 mo old and 20–30 g, were used in these experiments (n=24). Animals were anesthetized with 160 mg/kg intraperitoneally of pentobarbital. The proximal end of one femoral artery and distal portion of the corresponding saphenous artery were ligated, following which the artery, as well as all side-branches, were dissected free and excised. (All protocols were approved by St. Elizabeth's Institutional Animal Care and Use Committee.)

New Zealand White rabbits (3.8–4.2 kg, n=4, Pine Acre Rabbitry) were anesthetized with a mixture of ketamine (50 mg/kg) and acepromazine (0.8 mg/kg) following premedication with xylazine (2 mg/kg). After a longitudinal incision, the femoral artery was dissected free along its entire length; all branches of the femoral artery were also dissected free. After ligating the popliteal and saphenous arteries distally, the external iliac artery proximally and all femoral arterial branches, the femoral artery was completely excised (23). Isolation and Analysis

CD34 positive mononuclear blood cells (MB^{CD34+}) were isolated from peripheral blood by CD34 antibody-coated magnetic beads (Dynal) as described above.

FACS analysis indicated that 15.9±3.3% of selected cells versus <0.1% of the remaining cells expressed CD34. Depleted (MB^{CD34-}) cells were used as controls. Flk-1 antibody was used for magnetic bead selection of Flk-1 positive mononuclear blood cells (MB^{Flk1+}).

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MB^{CD34+} and MB^{CD34-} were plated separately in standard medium on tissue culture plastic, collagen type I, or fibronectin. When plated on tissue culture plastic or collagen at a density of $1\times10^3/mm^2$, a limited number of MB^{CD34+} attached, and became spindle shaped and proliferated for 4 wks. A subset of MB^{CD34+} plated on fibronectin promptly attached and became spindle shaped within 3 days (FIG. 1A); the number of attaching cells (AT^{CD34+}) in culture increased with time (FIG. 2). Attached cells were observed only sporadically among cultures of MB^{CD34-}, including cells followed for up to 4 wks on fibronectin-coated plates.

To confirm that spindle-shaped cells were derived from CD34 positive cells, MB^{CD34+} were labeled with the fluorescent dye, Dil, and co-plated with unlabeled MB^{CD34-} on fibronectin at an overall density of $5\times10^3/mm^2$; ratio of the two cell types was identical to that of the original mononuclear cell population (1% MB^{CD34+}, 99% MB^{CD34-}). Seven days later, Dil-labeled cells derived from MB^{CD34+}, initially accounting for only 1% of blood cells, accounted for $60.3\pm4.7\%$ of total attaching cells analyzed by FACS. Co-incubation with MB^{CD34-} increased proliferation to $>10\times MB^{CD34+}$ plated alone at a cell density of $5\times10/mm^2$ cell (d 3=131.3 ± 26.8 vs $9.7\pm3.5/mm^2$). MB^{CD34+}/MB^{CD34-} co-cultures also enhanced MB^{CD34+} differentiation, including formation of cellular networks and tube-like structures on fibronectin-coated plates (FIGS. 1B,C). These structures consisted principally of Dil-labeled MB^{CD34+} derived cells (FIG. 1C). Moreover, within 12 h of co-culture, multiple cluster formations were observed (FIG. 1D), consisting principally of Dil-labeled MB^{CD34+} derived cells (FIG. 1E). These clusters were comprised of round cells centrally, and sprouts of spindle-shaped cells at the periphery. The appearance and organization of these clusters resembled that of blood island-like cell clusters observed in dissociated quail epiblast culture, which induced ECs and gave rise to vascular structures in vitro (3). AT^{CD34+} at the cluster periphery were shown to take up Dil-labeled acetylated LDL, characteristic of EC lineage (13), whereas the round cells comprising the center of cluster did not (FIGS. 1F,G); the latter detached from the cluster several days later. Similar findings were observed in the experiments using MB^{Flk1+}.

Expression of Leukocyte and EC Markers

To further evaluate progression of MB^{CD34+} to an EC-like phenotype, cells were assayed for expression of leukocyte and EC markers. Freshly isolated MB^{CD34+} versus AT^{CD34+} cultured at densities of 1×10^3 cell/mm^2 for 7 days were incubated with fluorescent-labeled antibodies and analyzed by FACS (FIG. 3). Leukocyte common antigen, CD45, was identified on 94.1% of freshly isolated cells, but was essentially lost by 7 d in culture (FIG. 3). Augmented expression of UEA-1, CD34, CD31, Flk-1, Tie-2 and E-selectin—all denoting EC lineage (14)—was detected among AT^{CD34+} after 7 days in culture, compared to freshly isolated MB^{CD34+}. CD68 expression, suggesting monocyte/macrophage lineage, was limited to $6.0\pm2.4\%$ cells.

Expression of Factor VIII, UEA-1, CD31, ecNOS, and E-selectin was also documented by immunohistochemistry for AT^{CD34+} after 7 days culture (data not shown). After 3, 7, and 14 days in culture, more than 80% AT^{CD34+} took up Dil-labeled acLDL (13).

ECs uniquely express endothelial constitutive nitric oxide synthase (ecNOS). Accordingly, MB^{CD34+}, MB^{CD34-} and AT^{CD34+} were investigated for expression of ecNOS by RT-PCR (15). ecNOS mRNA was not detectable among $MBcD^{34-}$ and was present at very low levels in freshly isolated MB^{CD34+} (FIG. 4). In AT^{CD34+} cultured for 7 d, however, ecNOS mRNA was markedly increased (FIG. 5).

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Functional evidence of ecNOS protein in AT^{CD34+} was documented by measurement of nitric oxide in response to the EC-dependent agonist, acetylcholine (Ach), and the EC-specific mitogen, vascular endothelial growth factor (VEGF) (16) (FIG. 5); the latter parenthetically constitutes evidence for a functional Flk-1 receptor as well among AT^{CD34+}.

Cell-Cell Interaction

Cell-cell interaction is considered to play a decisive role in cell signaling, differentiation, and proliferation during hematopoiesis (19) and angiogenesis (20). To study the impact of MB^{CD34+} interaction with mature ECs on the differentiation of MB^{CD34+} into an EC-like phenotype, Dil-labeled MB^{CD34+} were plated on a confluent HUVEC monolayer. Adherent, labeled cells were found throughout the culture within 12 h (FIG. 6A), and increased in number for up to 3 d (FIG. 6B). When incubated with 50 ng/ml VEGF and 10 ng/ml bFGF, a meshwork of cord-like structures comprised of both Dil-labeled and unlabeled cells could be seen within 3 d after co-culture (FIG. 6C). Both cell types were then re-seeded on Matrigel (Becton Dickinson) coated slides and within 12 h demonstrated formation of capillary networks comprised of Dil-labeled MB^{CD34+} derived cells and HUVECs (FIG. 6D). To facilitate cell-cell interaction, HUVECs were pre-treated with TNF-α_(21),_resulting in increased numbers of AT^{CD34+} (FIG. 6E); synergistic augmentation was observed upon co-incubation with VEGF. Identically treated co-cultures of HUVECs and Dil-labeled MB^{CD34-} yielded desquamated labeled cells and/or no cords. Similar findings were observed when EC precursors were isolated using MB^{Flk1+}.

In Vivo Angiogenesis

Previous studies have established that ECs constitute the principal cell responsible for in vivo angiogenesis (1). To determine if MB^{CD34+} can contribute to angiogenesis in vivo, we employed two previously characterized animal models of hindlimb ischemia. For administration of human MB^{CD34+}, C57BL/6Jx129/SV background athymic nude mice were employed to avoid potential graft—versus host complications. Two days later, when the limb was severely ischemic, mice were injected with 5×10^5 Dil-labeled human MB^{CD34+} or MB^{CD34-} via the tail vein. Histologic sections of limbs examined 1, 2, 4, and 6 wks later for the presence of Dil labeled cells revealed numerous Dil-labeled cells in the neo-vascularized ischemic hindlimb. Labeled cells were more numerous in MB^{CD34+} versus MB^{CD34-} injected mice, and almost all labeled cells appeared to be integrated into capillary vessel walls (FIG. 8A,C,E,G).

No labeled cells were observed in the uninjured limbs of either MB^{CD34+} or MB^{CD34-} injected mice. Dil labeled cells were also consistently co-labeled with immunostains for UEA-1 lectin (FIG. 8B), CD31 (FIG. 8D), and Tie-2 (FIG. 8F). In contrast, in hindlimb sections from mice injected with MB^{CD34-}, labeled cells were typically found in stroma near capillaries, but did not form part of the vessel wall, and did not label with UEA-1 or anti-CD31 antibodies (FIG. 8G,H).

A transgenic mouse overexpressing β-galactosidase was then used to test the hypothesis that homologous grafts of EC progenitors could contribute to neovascularization in vivo. Flk-1 cell isolation was used for selection of EC progenitors due to lack of a suitable anti-mouse CD34 antibody. Approximately 1×10^4 MB^{Flk1+} were isolated from whole blood of 10 β-galactosidase transgenic mice with B6, 129 genetic background. MB^{Flk1+} or the same number of MB^{Flk1-} were injected into B6, 129 mice with hindlimb ischemia of 2 days duration. Immunostaining of ischemic

tissue for β-galactosidase, harvested 4 wks after injection, demonstrated incorporation of cells expressing β-galactosidase in capillaries and small arteries (FIG. 8I); these cells were identified as ECs by staining with anti-CD31 antibody and BS-1 lectin.

Finally, in vivo incorporation of autologous MB^{CD34+} into foci of neovascularization was tested in a rabbit model of unilateral hindlimb ischemia. MB^{CD34+} were isolated from 20 ml of blood obtained by direct venipuncture of normal New Zealand white rabbits immediately prior to surgical induction of unilateral hindlimb ischemia. Immediately following completion of the operative procedure, freshly isolated autologous Dil-labeled MB^{CD34+} were re-injected into the ear vein of the same rabbit from which the blood had been initially obtained. Four wks after ischemia, histologic sections of the ischemic limbs were examined. Dil-labeled cells were localized exclusively to neovascular zones of the ischemic limb, incorporated into capillaries and consistently expressing CD31 and UEA-1 (FIG. 8J,K).

Consistent with the notion that HSCs and ECs are derived from a common precursor, our findings suggest that under appropriate conditions, a subpopulation of MB^{CD34+} or MB^{Flk-1+} can differentiate into ECs in vitro. Moreover, the in vivo results suggest that circulating MB^{CD34+} or MB^{Flk1+} in the peripheral blood may constitute a contingency source of ECs for angiogenesis. Incorporation of in situ differentiating EC progenitors into the neovasculature of these adult species is consistent with vasculogenesis, a paradigm otherwise restricted to embryogenesis (2,3). The fact that these cells do not incorporate into mature blood vessels not undergoing angiogenesis suggests that injury, ischemia, and/or active angiogenesis are required to induce in situ differentiation of MB^{CD34+} to ECs.

EXAMPLE II

EC Progenitors Augment Reendothelialization

Following balloon injury, a denuded rat carotid artery was immediately excised and placed in culture in HUVEC medium, and Dil labeled CD34+ EC progenitor cells were seeded onto the artery. After 1 wk, the artery was washed with PBS to remove non-adherent cells. Consistent with the ability of CD34+ cells to differentiate into filtrating cells, Dil labeled cells were found within the smooth muscle cell layer of the artery.

Scanning electron microscopy of the intimal surface, however, showed that Dil-labeled cells also had adhered to the denuded arterial surface, assuming a morphology suggestive of ECs (FIG. 9). Dil labeled cells also incorporated into the capillary-like sprouts at the bare ends of the excised arterial segment, suggesting that CD34+ cells may be capable of participating in angiogenesis as well.

To determine if exogenously administered CD34+ EC progenitor cells can contribute to reendothelializationr of a denuded arterial surface in vivo, freshly isolated human CD34+ or CD34− cells were Dil labeled and seeded onto a denuded carotid artery of a nude rat. Following balloon denudation, 1.0×10^6 labeled cells in PBS was introduced into the denuded artery via a 22 G catheter, which remained in the artery for 30 min before the needle was withdrawn. The external carotid artery was then ligated, the common and internal carotid arterial ligatures removed, and the incision closed. The next day the rat was anesthetized and the vasculature perfusion fixed with Histo Choice (Amresco). The denuded arterial segment was excised and examined for the presence of adherent Dil labeled cells, which were identified in arteries seeded with CD34+ cells, but not CD34− cells.

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ciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements thereon without departing from the spirit and scope of the invention as set forth in the claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

   (iii) NUMBER OF SEQUENCES: 2

(2) INFORMATION FOR SEQ ID NO:1:

   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 30 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AAGACATTTT CGGGCTCACG CTGCGCACCC   30

(2) INFORMATION FOR SEQ ID NO:2:

   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 30 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TGGGGTAGGC ACTTTAGTAG TTCTCCTAAC   30

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This invention has been described in detail including the preferred embodiments thereof. However, it will be appre-

What is claimed is:

1. A method for inducing the formation of new blood vessels in an ischemic tissue in a patient in need thereof, comprising:

   administering to said patient host an effective amount of an isolated endothelial progenitor cell to induce new blood vessel formation in said ischemic tissue, wherein said endothelial progenitor cell are CD34$^+$, flk-1$^+$ or tie-2$^+$.

2. The method of claim 1, further comprising the step of administering to the patient an endothelial cell mitogen or a nucleic acid encoding an endothelial cell mitogen.

3. The method of claim 2, wherein the endothelial cell mitogen is selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor a and β, platelet-derived endothelial growth factor, platelet-derived growth factor, tumor necrosis factor α, hepatocyte growth factor, insulin like growth factor, erythropoietin, colony stimulating factor, macrophage-CSF, granulocyte/macrophage CSF and nitric oxidesynthase.

4. The method of claim 3, wherein the endothelial cell mitogen is vascular endothelial growth factor.

5. The method of claim 1, wherein said patient is in need of treatment for cerebrovascular ischemia, renal ischemia, pulmonary ischemia, limb ischemia, ischemic cardiomyopathy and myocardial ischemia.

6. A method of enhancing blood vessel formation in a patient in need thereof, comprising:

   a. selecting the patient in need thereof;

   b. isolating endothelial progenitor cells from the patient, wherein said endothelial progenitor cell are CD34$^+$, flk-1$^+$ or tie-2$^+$; and

c. readministering the endothelial progenitor cells to the patient.

7. A method for treating an injured blood vessel in a patient in need thereof, comprising:

a. selecting the patient in need thereof; and

b. isolating endothelial progenitor cells from the patient, wherein said endothelial progenitor cell are CD34$^+$, flk-1$^+$ or tie-2$^+$; and

c. readministering the endothelial progenitor cells to the patient.

8. The method of claim 7, wherein the injury is the result of balloon angioplasty.

9. The method of claim 7, wherein the injury is the result of deployment of an endovascular stent.

10. The method of claim 7, further comprising the step of administering to the patient an endothelial cell mitogen or a nucleic acid encoding an endothelial cell mitogen.

11. The method of claim 10, wherein the endothelial cell mitogen is selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor a and β, platelet-derived endothelial growth factor, platelet-derived growth factor, tumor necrosis factor α, hepatocyte growth factor, insulin like growth factor, erythropoietin, colony stimulating factor, macrophage-CSF, granulocyte/macrophage CSF and nitric oxidesynthase.

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EVIDENCE APPENDIX

ITEM NO. 15

Asahara, et al., 1997 <u>Science</u> article entitled,
"Isolation of Putative Progenitor Endothelial Cells for
Angiogenesis" cited by Appellant as Exhibit A
in Appeal Brief filed October 14, 2008

Isolation of Putative Progenitor Endothelial Cells for Angiogenesis

Takayuki Asahara, Toyoaki Murohara, Alison Sullivan,
Marcy Silver, Rien van der Zee, Tong Li,
Bernhard Witzenbichler, Gina Schatteman, Jeffrey M. Isner*

Putative endothelial cell (EC) progenitors or angioblasts were isolated from human peripheral blood by magnetic bead selection on the basis of cell surface antigen expression. In vitro, these cells differentiated into ECs. In animal models of ischemia, heterologous, homologous, and autologous EC progenitors incorporated into sites of active angiogenesis. These findings suggest that EC progenitors may be useful for augmenting collateral vessel growth to ischemic tissues (therapeutic angiogenesis) and for delivering anti- or pro-angiogenic agents, respectively, to sites of pathologic or utilitarian angiogenesis.

Postnatal neovascularization is thought to result exclusively from the proliferation, migration, and remodeling of fully differentiated ECs derived from preexisting blood vessels (1). This adult paradigm, referred to as angiogenesis, contrasts with vasculogenesis, the term applied to the formation of embryonic blood vessels from EC progenitors, or angioblasts (2).

Vasculogenesis begins as a cluster formation, or blood island, comprising angioblasts at the periphery and hematopoietic stem cells (HSCs) at the center (3). In addition to this spatial association, angioblasts and HSCs share certain antigenic determinants, including Flk-1, Tie-2, and CD34. Conceivably, then, these progenitor cells may derive from a common precursor (3, 4).

The demonstration that HSCs from peripheral blood can provide sustained hematopoietic recovery is inferential evidence for circulating stem cells (5). Here, we have investigated the hypothesis that peripheral blood contains cells that can differentiate into ECs (6). We exploited two antigens that are shared by angioblasts and HSCs to isolate putative angioblasts from the leukocyte fraction of peripheral blood. CD34 is expressed by all HSCs but is lost by hematopoietic cells as they differentiate (7). It is also expressed by many including most activated ECs in the adult (8). Flk-1, a receptor for vascular endothelial growth factor (VEGF) (9), is also expressed by both early HSCs and ECs but ceases to be expressed during hematopoietic differentiation (10, 11).

CD34-positive mononuclear blood cells (MB^CD34+) were isolated from human peripheral blood by means of magnetic beads

coated with antibody to CD34 (Dynal, Lake Success) (12). Fluorescence-activated cell sorting (FACS) analysis (13) indicated that 15.7 ± 3.3% of selected cells compared with <0.1% of the remaining cells expressed CD34. CD34-depleted cells (MB^CD34−) were used as controls. An antibody to Flk-1 was used for magnetic bead selection of Flk-1–positive mononuclear

blood cells (MB^Flk1+); among MB^Flk1+ cells, 20.0 ± 3.3% were Flk-1 positive.

The MB^CD34+ and MB^CD34− cells were plated separately (14) on tissue culture plastic, collagen type I, or fibronectin. When plated on tissue culture plastic or collagen at a density of 1 × 10^5 cells/mm^2, a limited number of MB^CD34+ attached, became spindle shaped, and proliferated for 4 weeks. A subset of MB^CD34+ plated on fibronectin promptly attached and became spindle shaped within 3 days (Fig. 1A); the number of attaching cells (AT^CD34+) in culture increased with time (probability P < 0.05, by analysis of variance) (Fig. 1B). Attached cells were observed only sporadically among MB^CD34− cultures, including cells followed for up to 4 weeks on fibronectin-coated plates.

To confirm that the spindle-shaped cells were derived from CD34-positive cells, we labeled MB^CD34+ cells with the fluorescent dye DiI and coplated them with unlabeled MB^CD34− cells on fibronectin at an overall density of 5 × 10^5 cells/mm^2; the ratio of the two cell types was identical to that of the original mononuclear cell population (1% MB^CD34+, 99% MB^CD34−). After 7 days, DiI-labeled cells derived from the MB^CD34+ culture, which initially account-

Fig. 1. Attachment, cluster formation, and capillary network development by progenitor ECs in vitro. (A) Spindle-shaped attaching cells (AT^CD34+) 7 days after plating MB^CD34+ (50 cells/mm^2) on fibronectin in standard medium (14). (B) Number of AT^CD34+ cells 12 hours and 3 days after culture of MB^CD34+ on plastic alone (CD34+/non), collagen coating (CD34+/Col), or fibronectin (CD34+/Fn), and MB^CD34− on fibronectin (CD34−/Fn). Network formation (C) and cord-like structures (D) were observed 48 hours after plating coculture of MB^CD34+, labeled with DiI, with unlabeled MB^CD34− cells (ratio of 1:100) on fibronectin. At 12 hours after coculture, MB^Flk1+-derived cells had formed multiple clusters (E and F). After 5 days, uptake of acLDL-DiI was detected in AT^CD34+ cells at the periphery but not the center of the cluster (G and H).
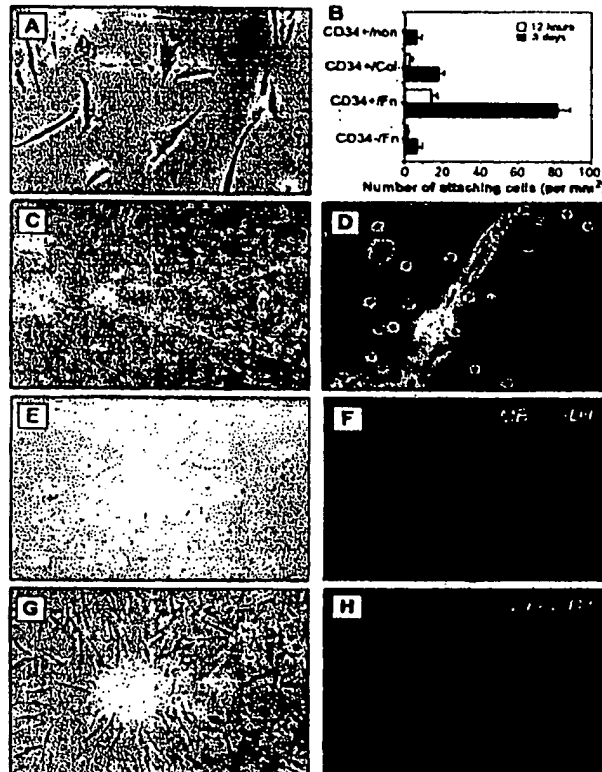
ed for only 1% of the blood cells, accounted for 60.3 ± 4.7% of total attaching cells as analyzed by FACS. Coincubation with MB^CD34− cells increased the proliferation rate to more than 10 times that of MB^CD34+ plated alone. Cocultures of MB^CD34+ and MB^CD34− cells also showed enhanced MB^CD34+ differentiation, including the formation of cellular networks and tube-like structures on fibronectin-coated plates (Fig. 1, C and D). These structures consisted principally of Dil-labeled MB^CD34+-derived cells (Fig. 1D). Furthermore, within 12 hours of coculture, multiple clusters had formed (Fig. 1E) that contained mostly MB^CD34+-derived cells (Fig. 1F). These clusters comprised round cells centrally and sprouts of spindle-shaped cells at the periphery. The appearance and organization of these clusters resembled that of blood island-like cell clusters observed in dissociated quail epiblast culture, which gave rise to ECs and vascular structures in vitro (3). AT^CD34+ cells at the cluster periphery took up Dil-labeled acetylated low density lipoprotein (acLDL), whereas the round cells did not (Fig. 1, G and H); the latter detached from the cluster several days later. The MB^Flk1+ cells behaved similarly.

To evaluate whether MB^CD34+ cells progressed to an EC-like phenotype, we assayed them for the expression of leukocyte and EC markers. Freshly isolated MB^CD34+ cells, AT^CD34+ cells cultured on fibronectin for 7 days, and human umbilical vein endothelial cells (HUVECs) were incubated with fluorescent-labeled antibodies and analyzed by FACS (Fig. 2). Leukocyte common antigen CD45 was identified on 94.1% of freshly

isolated cells but disappeared after 7 days of culture (Fig. 2). In freshly isolated MB^CD34+ cells, 15.7 ± 3.3% were CD34+, 27.6 ± 4.3% were Flk-1+, and 10.8 ± 0.9% were CD34+,Flk-1+. Expression of CD34, CD31, Flk-1, Tie-2, and E selectin—all markers of the EC lineage (11, 15)—was greater in AT^CD34+ cells after 7 days of culture than in freshly isolated MB^CD34+ cells.

Additional analyses (16) of AT^CD34+ cells after 7 days of culture showed limited (6.0 ± 2.4% cells) expression of CD68, a marker of the monocyte-macrophage lineage; positive immunostaining for factor VIII, ulex europaeus agglutinin-1 (UEA-1), CD31, endothelial constitutive nitric oxide synthase (ecNOS), and E selectin; and more than 80% uptake of Dil-labeled acLDL.

To confirm an EC-like phenotype of AT^CD34+ cells, we documented expression of ecNOS, Flk-1/KDR (Flk-1 is also known as VEGFR-2 in mouse, and KDR is the human homolog of VEGFR-2), and CD31 mRNA at 7, 14, and 21 days by reverse transcription-polymerase chain reaction (RT-PCR) (Fig. 3A). Evidence for ecNOS and Flk-1/KDR in AT^CD34+ cells was also demonstrated in a functional assay. Nitric oxide was produced in the cells in response to the EC-dependent agonist acetylcholine (Ach) and the EC-specific mitogen VEGF (Fig. 3B); the latter response also confirms that the cells express a functional Flk-1 receptor (17).

To determine if MB^CD34+ cells contribute to angiogenesis in vivo, we used mouse and rabbit models of hindlimb ischemia. For administration of human MB^CD34+ cells, C57BL/6J × 129/SV background athymic

nude mice were used to avoid potential graft-versus-host complications. Two days after creating unilateral hindlimb ischemia by excising one femoral artery, we injected mice with 5 × 10^5 Dil-labeled human MB^CD34+ or MB^CD34 cells into the tail vein. Histologic examination 1 to 6 weeks later revealed numerous (Fig. 4A) including proliferative (Fig. 4, C and D) Dil-labeled cells in the neovascularized ischemic hindlimb. Nearly all labeled cells appeared integrated into capillary vessel walls. In MB^CD34+-injected mice, 13.4 ± 5.7% of all CD31-positive capillaries contained Dil-labeled cells, compared with 1.6 ± 0.8% in MB^CD34−-injected mice (18). By 6 weeks, Dil-labeled cells were clearly arranged into capillaries among preserved muscle structures (Fig. 4, I and J).

No labeled cells were observed in the uninjured limbs of either MB^CD34+- or MB^CD34−-injected mice. Dil-labeled cells consistently colocalized with cells immunostained for CD31 (Fig. 4, B, F, and J), Tie-2 (Fig. 4G), and UEA-1 lectin (16). In contrast, in hindlimb sections from mice injected with MB^CD34−, Dil-labeled cells were typically found in stroma near capillaries, but they did not form part of the vessel wall nor did they colocalize with cells that stained with antibodies to either UEA-1 or CD31 (Fig. 4, K and L).

In a second set of mouse experiments, 1 × 10^4 MB^Flk1+ cells were isolated from

A

B

Fig. 3. Progenitor ECs express ecNOS, Flk-1/KDR, and CD31 mRNA and release NO. (A) Complementary DNA (from 10^6 cells) was amplified by PCR (40 cycles) with paired primers (23) (B) NO release from AT^CD34+ and AT^CD34− cells cultured in six-well plates was measured as described (24). NO production was measured in a well with incremental doses of VEGF and Ach. HUVECs and bovine aortic ECs were used as positive controls, and human coronary smooth muscle cells (HCSMCs) as negative control. The values are means ± SEM of 10 measurements for each group.

Fig. 2. FACS analysis of freshly isolated MB^CD34+ and AT^CD34+ cells after 7 days in culture, and HUVECs. Cells were labeled with fluorescent antibodies to CD45 (DAKO, Carpinteria); CD34, CD31 (Biodesign); Flk-1, Tie-2 (Santa Cruz); and E selectin (DAKO). Similar results were obtained in three or more experiments. The shaded area of each box denotes negative antigen gate, and the white area denotes positive gate. Numbers are the mean ± SEM percentage of cells for all experiments determined by comparison with corresponding negative control labeling.

whole blood of 10 transgenic mice constitutively overexpressing β-galactosidase (β-Gal) (all mice were Flk-1$^{+/+}$). MB^{Flk1+} or MB^{Flk1-} cells were injected into nontransgenic mice of the same genetic background that had hindlimb ischemia of 2 days duration. Immunostaining of ischemic tissue, harvested 4 weeks after injection, for β-Gal demonstrated incorporation of cells expressing β-Gal in capillaries and small arteries (Fig. 4M); these cells were identified as ECs by staining with antibody to CD31 (anti-CD31) and BS-1 lectin.

In vivo incorporation of autologous MB^{CD34+} cells into foci of neovascularization was also tested in a rabbit model of unilateral hindlimb ischemia. MB^{CD34+} cells were isolated from 20 ml of blood obtained by direct venipuncture of normal New Zealand White rabbits immediately before surgical induction of unilateral hindlimb ischemia (19). Immediately after surgery, freshly isolated autologous DiI-labeled MB^{CD34+} were reinjected into the ear vein of the same rabbit. Histologic examination of the ischemic limbs 4 weeks later revealed that DiI-labeled cells were localized exclusively to neovascular zones of the ischemic limb (Fig. 4, N and O) and were incorporated into 9.7 ± 4.5% of the capillaries that
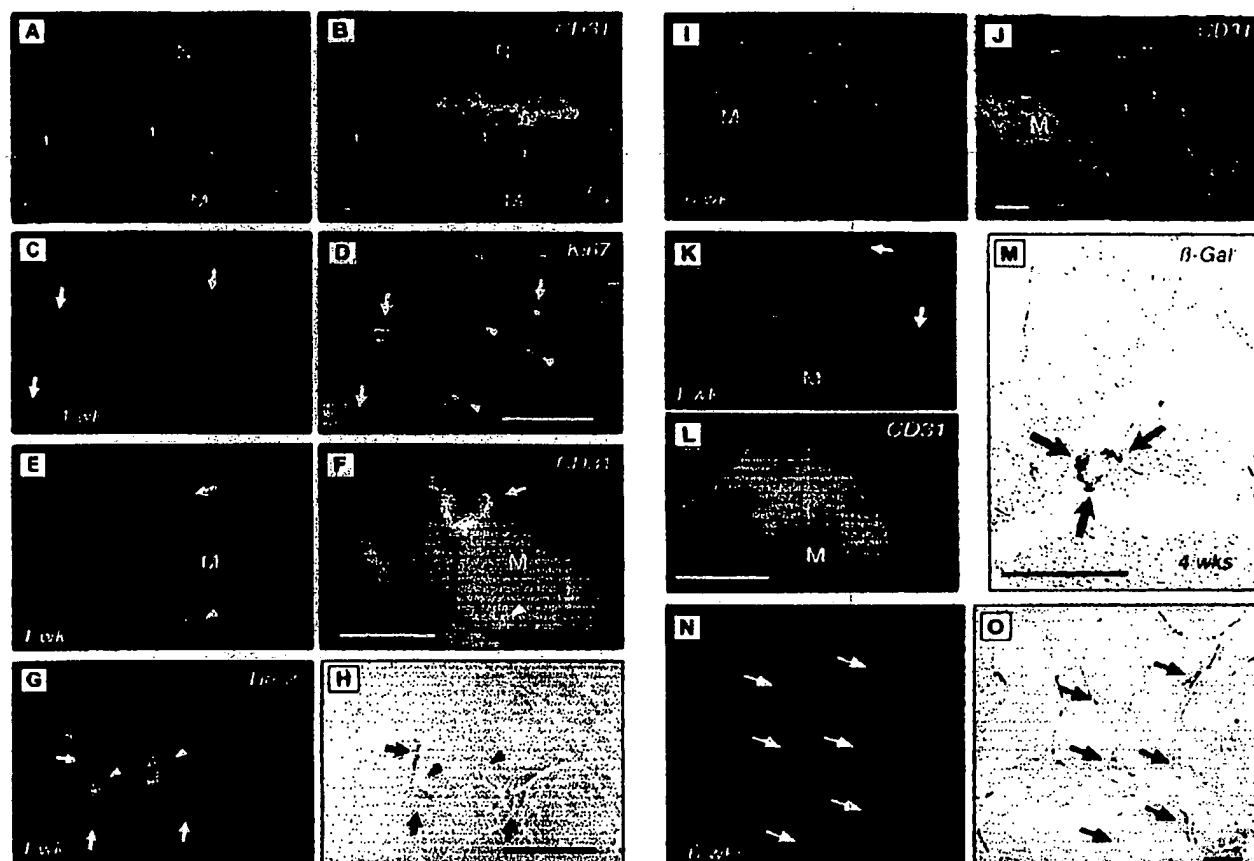
Fig. 4. Heterologous (panels A to L), homologous (M), or autologous (panels N and O) EC progenitors incorporate into sites of angiogenesis in vivo. (A and B) DiI-labeled MB^{CD34+} (red, arrows) between skeletal myocytes (M), including necrotic (N) myocytes 1 week after injection; most are colabeled with CD31 (green, arrows). Note a preexisting artery (A), identified as CD31-positive, but DiI-negative. (C and D) Evidence of proliferative activity among several DiI-labeled MB^{CD34+}-derived cells (red, arrows), indicated by coimmunostaining for antibody to Ki67 (Vector Lab, Burlingame, California) (green). Proliferative activity is also seen among DiI-negative, Ki67-positive capillary ECs (arrowheads); both cell types contribute to neovasculature. (E) DiI (red) and CD31 (green) in capillary ECs (arrows in E and F) between skeletal myocytes, photographed through a double filter 1 week after DiI-labeled MB^{CD34+} injection. (F) A single green filter shows CD31 (green) expression in DiI-labeled capillary ECs integrated into the capillary with native (DiI-negative, CD31-positive) ECs (arrowheads in E and F). (G) Immunostaining 1 week after MB^{CD34+} injection showing capillaries comprising DiI-labeled MB^{CD34+}-derived cells expressing Tie-2 receptor (green). Several MB^{CD34+}-derived cells (arrows) Tie-2 positive and integrated with some Tie-2–positive host capillary cells (arrowheads) identified by the absence of fluorescence. (H) Phase-contrast photomicrograph of the same section shown in (G) indicates the corresponding DiI-labeled (arrows) and -unlabeled (arrowheads) capillary ECs. (I and J) Six weeks after administration. MB^{CD34+}-derived cells (red, arrows) colabel for CD31 in capillaries between preserved skeletal myocytes (M). (K and L) One week after injection of MB^{CD34+}-, isolated MB^{CD34+}-derived cells (red, arrows) are observed between myocytes but do not express CD31. (M) Immunostaining of β-Gal in a tissue section harvested from ischemic muscle of C57BL/6J,129/SV mice 4 weeks after the administration of MB^{Flk1+} isolated from transgenic mice constitutively expressing β-Gal. (Flk-1 cell isolation was used for selection of EC progenitors because of the lack of a suitable antibody to mouse CD34.) Cells overexpressing β-Gal (arrows) were incorporated into capillaries and small arteries; these cells were identified as ECs by anti-CD31 and BS-1 lectin (16). (N and O) Section of muscle harvested from rabbit ischemic hindlimb 4 weeks after administration of autologous MB^{CD34+} cells. Red fluorescence in (N) indicates localization of MB^{CD34+}-derived cells in capillaries seen (arrows) in the phase-contrast photomicrograph in (O). Each scale bar is 50 μm.

consistently expressed CD31 and reacted with BS-1 lectin.

In summary, our findings suggest that cells isolated with anti-CD34 or anti-Flk-1 can differentiate into ECs in vitro. The in vivo results suggest that circulating MB^{CD34+} or MB^{Flk1+} cells may contribute to neuangiogenesis in adult species, consistent with vasculogenesis, a paradigm otherwise restricted to embryogenesis (2, 3). A potentially limiting factor in strategies designed to promote neovascularization of ischemic tissues (20) is the resident population of ECs that is competent to respond to administered angiogenic cytokines (21). This issue may be successfully addressed with autologous EC transplants. The fact that progenitor ECs home to foci of angiogenesis suggests potential utility as autologous vectors for gene therapy. For antineoplastic therapies, MB^{CD34+} cells could be transfected with or coupled to antitumor drugs or angiogenesis inhibitors. For treatment of regional ischemia, angiogenesis could be amplified by transfection of MB^{CD34+} cells to achieve constitutive expression of angiogenic cytokines or provisional matrix proteins or both (22).

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12. Single donor human peripheral blood was obtained with a 20-gauge intravenous catheter. The first 3 ml was discarded, and the leukocyte fraction was obtained by Ficoll density gradient centrifugation. The cells were plated on plastic tissue culture for 1 hour to avoid contamination by differentiated adhesive cells.
13. MB^{CD34+}, MB^{CD34-}, and MB^{Flk1+} cells (>1 × 10^6 of each) were analyzed with anti-CD34 (Biodesign, Kennebunkport, ME) and anti-Flk-1 (Santa Cruz Biotechnologies, Santa Cruz, CA).
14. The medium for all cell culture experiments was M-199 with 20% fetal bovine serum and bovine brain

extract (Clonetics, San Diego).
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18. The mean percent of DII-labeled capillaries among total CD31-positive capillaries was determined by averaging counts made in 10 randomly selected fields (×400).
19. New Zealand White rabbits (3.8 to 4.2 kg, n = 4, Pine Acre Rabbitry, Norton, MA) underwent ligation of the popliteal and saphenous arteries distally, the external iliac artery proximally, and all femoral arterial branches, after which the femoral artery was excised [S. Takeshita et al., J. Clin. Invest. 93, 662 (1994); L. Q. Pu et al., Circulation 68, 208 (1993); R. Baffour et al., J. Vasc. Surg. 16, 181 (1992)].
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23. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a positive control. The paired primers used (sense/antisense) were as follows: for ocNOS,

AAG ACA TTT TCG GGC TCA CGC TGC GCA CCC/ TGG GGT ACG CAC TTT AGT AGT TCT CCT AAC (548-base pairs (bp) PCR product); for Flk-1 (KDH), CAA CAA AGT CGG GAG AGG AG/ATG ACG ATG GAC AAG TAG CC (819-bp PCR product); for CD31, GCT GTT GGT GGA AGC AGT GC/GAA GTT GGC TGG AGG TGC TC (845-bp PCR product); for GAPDH, TGA AGG TCG GAG TCA ACG GAT TTG/ CAT GTG GGC CAT GAG GTC CAC CAC (983-bp PCR product).
24. NO release was measured with a NO-specific polarographic electrode connected to a NO meter (Iso-NO, World Precision Instruments, Sarasota, FL). AT^{CD34+} or AT^{CD34-} cells cultured in six-well plates were washed and then bathed in 5 ml of filtered Krebs-Henseleit solution. Cell plates were kept on a slide warmer (Lab Line Instruments, Melrose Park, IL) to maintain temperature between 35° and 37°C. The sensor probe was inserted vertically into the wells, and the tip of the electrode was positioned 2 mm under the surface of the solution.
25. Supported by grants from NIH National Heart, Lung, and Blood Institute numbers 02824, 53354, and 57516, the American Heart Association, the E. L. Wiegand Foundation, and in part by the Uehara Memorial Foundation (T.M.).

4 October 1996; accepted 14 January 1997

Somatic Frameshift Mutations in the BAX Gene in Colon Cancers of the Microsatellite Mutator Phenotype

Nicholas Rampino, Hiroyuki Yamamoto, Yurij Ionov, Yan Li, Hisako Sawai, John C. Reed, Manuel Perucho*

Cancers of the microsatellite mutator phenotype (MMP) show exaggerated genomic instability at simple repeat sequences. More than 50 percent (21 out of 41) of human MMP$^+$ colon adenocarcinomas examined were found to have frameshift mutations in a tract of eight deoxyguanosines [$(G)_8$] within BAX, a gene that promotes apoptosis. These mutations were absent in MMP$^-$ tumors and were significantly less frequent in $(G)_8$ repeats from other genes. Frameshift mutations were present in both BAX alleles in some MMP$^+$ colon tumor cell lines and in primary tumors. These results suggest that inactivating BAX mutations are selected for during the progression of colorectal MMP$^+$ tumors and that the wild-type BAX gene plays a suppressor role in a p53-independent pathway for colorectal carcinogenesis.

The MMP pathway for colon cancer is characterized by genomic instability that leads to the accumulation of deletion and insertion mutations at simple repeat sequences (1–3). The fixation of these slippage-induced replication errors as mutations (4) is associated with defects in DNA mismatch repair (5). Colorectal MMP$^+$ tumors frequently contain frameshift mutations in the type II transforming growth factor-β (TGF-β) receptor gene (6) but are usually wild type for the p53 tumor suppressor gene (1, 7). In addition to its central role in cell growth arrest (8), p53 also plays a role in apoptosis in response to DNA

damage (9). The p53 protein transactivates BAX (10), a member of the BCL2 gene family (11) that promotes apoptosis (12).

The human BAX gene contains a tract of eight consecutive deoxyguanosines in the third coding exon, spanning codons 38 to 41 (ATG GGG GGG GAG) (12). To determine whether this sequence is a mutational target in MMP$^+$ tumor cells, we amplified by the polymerase chain reaction (PCR) the region containing the $(G)_8$ tract from various MMP$^+$ tumor cell lines. This analysis revealed band shifts suggestive of insertions and deletions of one nucleotide in some of these tumor cells (Fig. 1A). Prostate (DU145) and colon (LS180) tumor cells exhibited PCR patterns indistinguishable from those amplified from plasmids containing a BAX fragment with mutant $(G)_9$ and $(G)_7$ tracts (Fig. 1A, P9 and P7),

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EVIDENCE APPENDIX

ITEM NO. 16

Nabel U.S. Patent No. 5,328,470
cited by Appellant as Reference AD
in the Information Disclosure Statement
filed February 15, 2001

United States Patent [19]

Nabel et al.

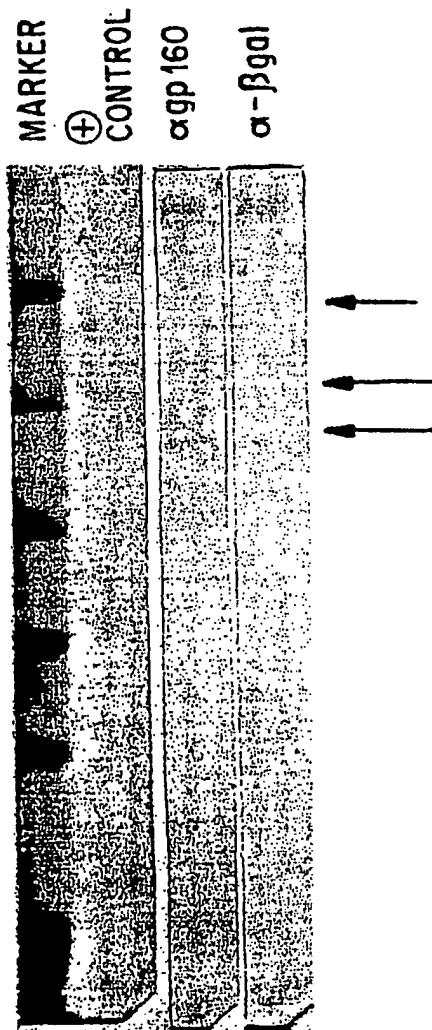[11] Patent Number: 5,328,470

[45] Date of Patent: Jul. 12, 1994

[54] **TREATMENT OF DISEASES BY SITE-SPECIFIC INSTILLATION OF CELLS OR SITE-SPECIFIC TRANSFORMATION OF CELLS AND KITS THEREFOR**

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[73] Assignee: **The Regents of the University of Michigan,** Ann Arbor, Mich.

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[57] **ABSTRACT**

A method for the direct treatment towards the specific sites of a disease is disclosed. This method is based on the delivery of proteins by catheterization to discrete blood vessel segments using genetically modified or normal cells or other vector systems. Endothelial cells expressing recombinant therapeutic agent or diagnostic proteins are situated on the walls of the blood vessel or in the tissue perfused by the vessel in a patient. This technique, provides for the transfer of cells or vectors and expression of recombinant genes in vivo and allows the introduction of proteins of therapeutic or diagnostic value for the treatment of diseases.

10 Claims, 4 Drawing Sheets
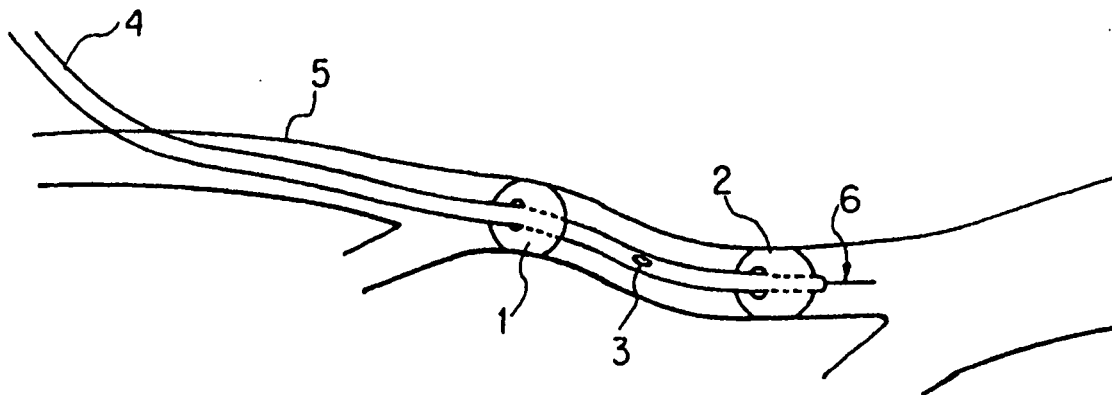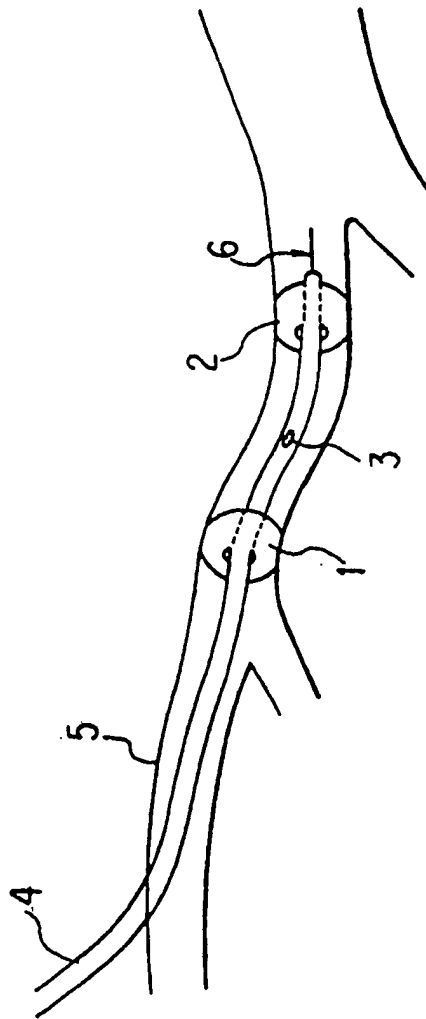
FIG.1
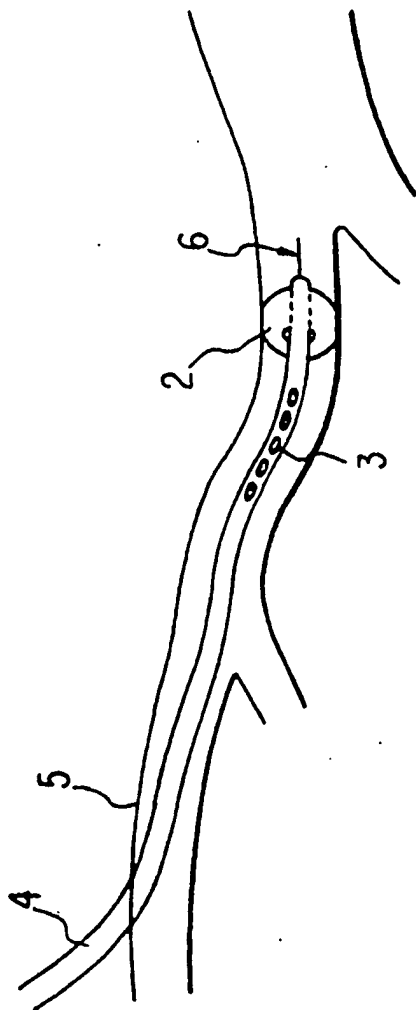
FIG.2

FIG. 3
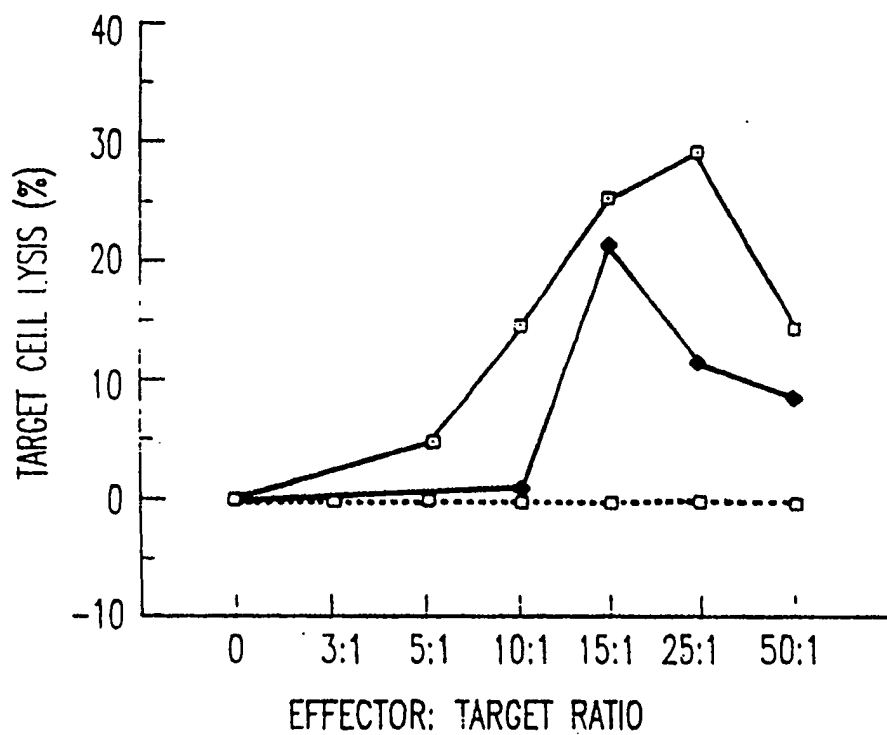
I.V. INJECTIONS

□——— SOLUTION C/H−2Ks

◆——— LIPOFECTIN / H−2Ks

□------ LIPOFECTIN / RSV−B−GAL

WESTERN BLOT ANALYSIS

FIG. 4

1

TREATMENT OF DISEASES BY SITE-SPECIFIC INSTILLATION OF CELLS OR SITE-SPECIFIC TRANSFORMATION OF CELLS AND KITS THEREFOR

This is a continuation-in-part of U.S. patent application Ser. No. 07/724,509, filed on Jun. 28, 1991, now pending, which is a continuation-in-part of U.S. patent application Ser. No. 07/331,336, filed on Mar. 31, 1989, now abandoned.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to the treatment of diseases by the site-specific instillation or transformation of cells and kits therefor. The present invention also relates to a method for modulating the immune system of an animal by the in vivo introduction of recombinant genes.

2. Discussion of the Background

The effective treatment of many acquired and inherited diseases remains a major challenge to modern medicine. The ability to deliver therapeutic agents to specific sites in vivo would an asset in the treatment of, e.g., localized diseases. In addition the ability to cause a therapeutic agent to perfuse through the circulatory system would be effective for the treatment of, e.g., inherited diseases and acquired diseases or cancers.

For example, it would be desirable to administer in a steady fashion an antitumor agent or toxin in close proximity to a tumor. Similarly, it would be desirable to cause a perfusion of, e.g., insulin in the blood of a person suffering from diabetes. However, for many therapeutic agents there is no satisfactory method of either site-specific or systemic administration.

In addition, for many diseases, it would be desirable to cause, either locally or systemically, the expression of a defective endogenous gene, the expression of a exogenous gene, or the suppression of an endogenous gene. Again, these remain unrealized goals.

In particular, the pathogenesis of atherosclerosis is characterized by three fundamental biological processes. These are: 1) proliferation of intimal smooth muscle cells together with accumulated macrophages; 2) formation by the proliferated smooth muscle cells of large amounts of connective tissue matrix; and 3) accumulation of lipid, principally in the form of cholesterol esters and free cholesterol, within cells as well as in surrounding connective tissue.

Endothelial cell injury is an initiating event and is manifested by interference with the permeability barrier of the endothelium, alterations in the nonthrombogenic properties of the endothelial surface, and promotion of procoagulant properties of the endothelium. Monocytes migrate between endothelial cells, become active as scavenger cells, and differentiate into macrophages.

Macrophages then synthesize and secrete growth factors including platelet derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), and transforming growth factor alpha (TGF-a). These growth factors are extremely potent in stimulating the migration and proliferation of fibroblasts and smooth muscle cells in the atherosclerotic plaque. In addition, platelets may interact with the injured endothelial cell and the activated macrophage to potentiate the elaboration of growth factors and thrombus formation.

2

Two major problems in the clinical management of coronary artery disease include thrombus formation in acute myocardial ischemia and restenosis following coronary angioplasty (PTCA). Both involve common cellular events, including endothelial injury and release of potent growth factors by activated macrophages and platelets. Coronary angioplasty produces fracturing of the atherosclerotic plaque and removal of the endothelium. This vascular trauma promotes platelet aggregation and thrombus formation at the PTCA site. Further release of mitogens from platelets and macrophages, smooth muscle cell proliferation and monocyte infiltration result in restenosis.

Empiric therapy with antiplatelet drugs has not prevented this problem, which occurs in one-third of patients undergoing PTCA. A solution to restenosis is to prevent platelet aggregation, thrombus formation, and smooth muscle cell proliferation.

Thrombus formation is also a critical cellular event in the transition from stable to unstable coronary syndromes. The pathogenesis most likely involves acute endothelial cell injury and/or plaque rupture, promoting dysjunction of endothelial cell attachment, and leading to the exposure of underlying macrophage foam cells. This permits the opportunity for circulating platelets to adhere, aggregate, and form thrombi.

The intravenous administration of thrombolytic agents, such as tissue plasminogen activator (tPA) results in lysis of thrombus in approximately 70% of patients experiencing an acute myocardial infarction. Nonetheless, approximately 30% of patients fail to reperfuse, and of those patients who undergo initial reperfusion of the infarct related artery, approximately 25% experience recurrent thrombosis within 24 hours. Therefore, an effective therapy for rethrombosis remains a major therapeutic challenge facing the medical community today.

As noted above, an effective therapy for rethrombosis is by far not the only major therapeutic challenge existing today. Others include the treatment of other ischemic conditions, including unstable angina, myocardial infarction or chronic tissue ischemia, or even the treatment of acquired and inherited diseases or cancers. These might be treated by the effective administration of anticoagulants, vasodilatory, angiogenic, growth factors or growth inhibitors to a patient. Thus, there remains a strongly felt need for an effective therapy in all of these clinical settings.

In addition, it is desirable to be able to modulate the immune system of an animal. In particular, much effort has been directed toward the development of vaccines to provide immunological protection from infection. However, the development of safe vaccines which can be readily administered to large numbers of patients is problematic, and for many diseases, such as, e.g., AIDS, no safe and effective vaccine is as yet available. Further, it is also sometimes desirable to specifically suppress an animals immune response to prevent rejection of a transplant. Efforts to suppress transplant rejection have resulted in the development of drugs which result in a general suppression of the immune response, rather than specific supression to transplantation antigens, and such drugs are not always effective. Thus, there remains a need for a method to modulate the immune system of an animal.

3

SUMMARY OF THE INVENTION

Accordingly, one object of the present invention is to provide a novel method for the site-specific administration of a therapeutic agent.

It is another object of the present invention to provide a method for the perfusion of a therapeutic agent in the blood stream of a patient.

It is another object of the present invention to provide a method for causing the expression of an exogenous gene in a patient.

It is another object of the present invention to provide a method for causing the expression of a defective endogenous gene in a patient.

It is another object of the present invention to provide a method for suppressing the expression of an endogenous gene in a patient.

It is another object of the present invention to provide a method for site-specifically replacing damaged cells in a patient.

It is another object of the present invention to provide a method for the treatment of a disease by causing either the site-specific administration of a therapeutic agent or the perfusion of a therapeutic agent in the bloodstream of a patient.

It is another object of the present invention to provide a method for the treatment of a disease by causing either the expression of an exogenous gene, the expression of a defective endogenous gene, or the suppression of the expression of an endogenous gene in a patient.

It is another object of the present invention to provide a method for the treatment of a disease by site-specifically replacing damaged cells in a patient.

It is another object of the present invention to provide a kit for site-specifically instilling normal or transformed cells in a patient.

It is another object of the present invention to provide a kit for site-specifically transforming cells in vivo.

It is another object of the present invention to provide a method for modulating the immune system of an animal.

It is another object of the present invention to provide a method for modulating the immune system of an animal to sensitize the animal to a foreign molecule.

It is another object of the present invention to provide a method to stimulate the immune system of an animal to reject proteins in order to protect against infection by a microorganism or virus.

It is another object of the present invention to provide a method for modulating the immune system of an animal to tolerize the animal to a foreign molecule.

It is another object of the present invention to provide a method for modulating the immune system of an animal to reduce the tendency to reject a transplant.

It is another object of the present invention to provide a novel kit for transforming cells by systemic administration in vivo.

These and other objects of this invention which will become apparent during the course of the following detailed description of the invention have been discovered by the inventors to be achieved by (a) a method which comprises either (i) site-specific instillation of either normal (untransformed) or transformed cells in a patient or (ii) site-specific transformation of cells in a patient and (b) a kit which contains a catheter for (i) site-specific instillation of either normal or transformed cells or (ii) site-specific transformation of cells.

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Site-specific instillation of normal cells can be used to replace damaged cells, while instillation of transformed cells can be used to cause the expression of either a defective endogenous gene or an exogenous gene or the suppression of an endogenous gene product. Instillation of cells in the walls of the patient's blood vessels can be used to cause the steady perfusion of a therapeutic agent in the blood stream.

The inventors have also discovered that by transforming cells of an animal, in vivo, it is possible to modulate the animal's immune system. In particular, by transforming cells of an animal, with a recombinant gene, by site-specific or systemic administration it is possible to modulate the animal's immune system to sensitize the animal to the molecule for which the recombinant gene encodes. Alternatively, by transforming cells of an animal with a recombinant gene, specifically at a site which determines the specificity of the immune system, such as, e.g., the thymus, it is possible to modulate the immune system of an animal to suppress the immune response to the molecule encoded by the recombinant gene.

BRIEF DESCRIPTION OF THE DRAWINGS

A more complete appreciation of the invention and many of the attendant advantages thereof will be readily obtained as the same become better understood by reference to the following detailed description when considered in connection with the accompanying figures, wherein:

FIGS. 1 and 2 illustrate the use of a catheter in accordance with the invention to surgically or percutaneously implant cells in a blood vessel or to transform in vivo cells present on the wall of a patient's blood vessel;

FIG. 3 illustrates the relationship between the % of target cell lysis and the effector:target ratio for CTL cells; and

FIG. 4 illustrates the results of a Western blot analysis.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Thus, in one embodiment, the present invention is used to treat diseases, such as inherited diseases, systemic diseases, diseases of the cardiovascular system, diseases of particular organs, or tumors by instilling normal or transformed cells or by transforming cells.

The cells which may be instilled in the present method include endothelium, smooth muscle, fibroblasts, monocytes, macrophages, and parenchymal cells. These cells may produce proteins which may have a therapeutic or diagnostic effect and which may be naturally occurring or arise from recombinant genetic material.

Referring now to the figures, wherein like reference numerals designate identical or corresponding parts throughout the several views, and more particularly to FIG. 1 thereof, this figure illustrates the practice of the present invention with a catheter having a design as disclosed in U.S. Pat. No. 4,636,195, which is hereby incorporated by reference. This catheter may be used to provide normal or genetically altered cells on the walls of a vessel or to introduce vectors for the local transformation of cells. In the figure, 5 is the wall of the blood vessel. The figure shows the catheter body 4 held in place by the inflation of inflatable balloon means 1 and 2. The section of the catheter body 4 situated between balloon means 1 and 2 is equipped with instillation port

means 3. The catheter may be further equipped with a guidewire means 6. FIG. 2 illustrates the use of a similar catheter, distinguished from the catheter illustrated in FIG. 1 by the fact that it is equipped with only a single inflatable balloon means 2 and a plurality of instillation port means 3. This catheter may contain up to twelve individual instillation port means 3, with five being illustrated.

In the case of delivery to an organ, the catheter may be introduced into the major artery supplying the tissue. Cells containing recombinant genes or vectors can be introduced through a central instillation port after temporary occlusion of the arterial circulation. In this way, cells or vector DNA may be delivered to a large amount of parenchymal tissue distributed through the capillary circulation. Recombinant genes can also be introduced into the vasculature using the double balloon catheter technique in the arterial circulation proximal to the target organ. In this way, the recombinant genes may be secreted directly into the circulation which perfuse the involved tissue or may be synthesized directly within the organ.

In one embodiment, the therapeutic agents are secreted by vascular cells supplying specific organs affected by the disease. For example, ischemic cardiomyopathy may be treated by introducing angiogenic factors into the coronary circulation. This approach may also be used for peripheral, vascular or cerebrovascular diseases where angiogenic factors may improve circulation to the brain or other tissues. Diabetes mellitus may be treated by introduction of glucose-responsive insulin secreting cells in the portal circulation where the liver normally sees a higher insulin concentration than other tissues.

In addition to providing local concentrations of therapeutic agents, the present method may also be used for delivery of recombinant genes to parenchymal tissues, because high concentrations of viral vector and other vectors can be delivered to a specific circulation. Using this approach, deficiencies of organ-specific proteins may also be treated. For example, in the liver, α-antitrypsin inhibitor deficiency or hyperchloresterolemia may be treated by introduction of α-antitrypsin or the LDL receptor gene. In addition, this approach may be used for the treatment of a malignancy. Secretion of specific recombinant toxin genes into the circulation of inoperable-tumors provides a therapeutic effect. Examples include acoustic neuromas or certain hemangiomas which are otherwise unresurrectable.

In clinical settings, these therapeutic recombinant genes are introduced in cells supplying the circulation of the involved organ. Although the arterial and capillary circulations are the preferred locations for introduction of these cells, venous systems are also suitable.

In its application to the treatment of local vascular damage the present invention provides for the expression of proteins which ameliorate this condition in situ. In one embodiment, because vascular cells are found at these sites, they are used as carriers to convey the therapeutic agents.

The invention thus, in one of its aspects, relies on genetic alteration of endothelial and other vascular cells or somatic cell gene therapy, for transmitting therapeutic agents (i.e., proteins, growth factors) to the localized region of vessel injury. To successfully use gene transplantation in the cells, four requirements must be fulfilled. First, the gene which is to be implanted into the cell must be identified and isolated. Second, the gene to

be expressed must be cloned and available for genetic manipulation. Third, the gene must be introduced into the cell in a form that will be expressed or functional. Fourth, the genetically altered cells must be situated in the vascular region where it is needed.

In accordance with the present invention the altered cells or appropriate vector may be surgically, percutaneously, or intravenously introduced and attached to a section of a patient's vessel wall. Alternatively, some of the cells existing on the patient's vessel wall are transformed with the desired genetic material or by directly applying the vector. In some instances, vascular cells which are not genetically modified can be introduced by these methods to replace cells lost or damaged on the vessel surface.

Any blood vessel may be treated in accordance with this invention; that is, arteries, veins, and capillaries. These blood vessels may be in or near any organ in the human, or mammalian, body.

Introduction of normal or genetically altered cells into a blood vessel

This embodiment of the invention may be illustrated as follows:

I. Establishment of endothelial or other vascular cells in tissue culture.

Initially, a cell line is established and stored in liquid nitrogen. Prior to cryopreservation, an aliquot is taken for infection or transfection with a vector, viral or otherwise, containing the desired genetic material.

Endothelial or other vascular cells may be derived enzymatically from a segment of a blood vessel, using techniques previously described in J. W. Ford, et al., In Vitro, 17, 40 (1981). The vessel is excised, inverted over a stainless steel rod and incubated in 0.1% trypsin in Ca++- and Mg++- free Hank's balanced salt solution (BSS) with 0.125% EDTA at pH 8 for 10 min at 37° C.

Cells (0.4 to 1.5×10^6) are collected by centrifugation and resuspended in medium 199 (GIBCO) containing 10% fetal bovine serum, endothelial cell growth supplement (ECGS, Collaborative Research, Waltham, Mass.) at 25 μg/ml, heparin at 15 U/ml, and gentamicin (50 μg/ml). Cells are added to a 75 cm² tissue culture flask precoated with gelatin (2 mg/ml in distilled water). Cells are fed every second day in the above medium until they reach confluence.

After two weeks in culture, the ECGS and heparin may be omitted from the medium when culturing porcine endothelium. If vascular smooth muscle cells or fibroblasts are desired the heparin and ECGS can be omitted entirely from the culturing procedure. Aliquots of cells are stored in liquid nitrogen by resuspending to approximately 10^6 cells in 0.5 ml of ice cold fetal calf serum on ice. An equal volume of ice cold fetal calf serum containing 10% DMSO is added, and cells are transferred to a prechilled screw cap Corning freezing tube. These cells are transferred to a −70° C. freezer for 3 hours before long term storage in liquid nitrogen.

The cells are then infected with a vector containing the desired genetic material.

II. Introduction of cells expressing normal or exogenous proteins into the vasculature.

A. Introduction of cells expressing relevant proteins by catheterization.

The patient is prepared for catheterization either by surgery or percutaneously, observing strict adherence to sterile techniques. A cutdown procedure is performed over the target blood vessel or a needle is in-

serted into the target blood vessel after appropriate anesthesia. The vessel (5) is punctured and a catheter, such as described in U.S. Pat. No. 4,636,195, which is hereby incorporated by reference (available from USCI, Billerica, Mass.) is advanced by guidewire means (6) under fluoroscopic guidance, if necessary, into the vessel (5) (FIG. 1). This catheter means (4) is designed to introduce infected endothelial cells into a discrete region of the artery. The catheter has a proximal and distal balloon means (2) and (1), respectively, (e.g., each balloon means may be about 3 mm in length and about 4 mm in width), with a length of catheter means between the balloons. The length of catheter means between the balloons has a port means connected to an instillation port means (3). When the proximal and distal balloons are inflated, a central space is created in the vessel, allowing for instillation of infected cells though the port.

A region of the blood vessel is identified by anatomical landmarks and the proximal balloon means (2) is inflated to denude the endothelium by mechanical trauma (e.g., by forceful passage of a partially inflated balloon catheter within the vessel) or by mechanical trauma in combination with small amounts of a proteolytic enzyme such as dispase, trypsin, collagenase, papain, pepsin, chymotrypsin or cathepsin, or by incubation with these proteolytic enzymes alone. In addition to proteolytic enzymes, liposes may be used. The region of the blood vessel may also be denuded by treatment with a mild detergent or the like, such as NP-40, Triton X100, deoxycholate, or SDS.

The denudation conditions are adjusted to achieve essentially complete loss of endothelium for cell transfers or approximately 20 to 90%, preferably 50 to 75%, loss of cells from the vessel wall for direct infection. In some instances cell removal may not be necessary. The catheter is then advanced so that the instillation port means (3) is placed in the region of denuded endothelium. Infected, transfected or normal cells are then instilled into the discrete section of artery over thirty minutes. If the blood vessel is perfusing an organ which can tolerate some ischemia, e.g., skeletal muscle, distal perfusion is not a major problem, but can be restored by an external shunt if necessary, or by using a catheter which allows distal perfusion. After instillation of the infected endothelial cells, the balloon catheter is removed, and the arterial puncture site and local skin incision are repaired. If distal perfusion is necessary, an alternative catheter designed to allow distal perfusion may be used.

B. Introduction of recombinant genes directly into cells on the wall of a blood vessel or perfused by a specific circulation in vivo; infection or transfection of cells on the vessel wall and organs.

Surgical techniques are used as described above. Instead of using infected cells, a high titer desired genetic material transducing viral vector (10^5 to 10^6 particles/ml) or DNA complexed to a delivery vector is directly instilled into the vessel wall using the double balloon catheter technique. This vector is instilled in medium containing serum and polybrene (10 μg/ml) to enhance the efficiency of infection. After incubation in the dead space created by the catheter for an adequate period of time (0.2 to 2 hours or greater), this medium is evacuated, gently washed with phosphate-buffered saline, and arterial circulation is restored. Similar protocols are used for post operative recovery.

The vessel surface can be prepared by mechanical denudation alone, in combination with small amounts of proteolytic enzymes such as dispase, trypsin, collagenase or cathepsin, or by incubation with these proteolytic enzymes alone. The denudation conditions are adjusted to achieve the appropriate loss of cells from the vessel wall.

Viral vector or DNA-vector complex is instilled in Dulbecco's modified Eagle's medium using purified virus or complexes containing autologous serum, and adhesive molecules such as polybrene (10 μg/ml), poly-L-lysine, dextran sulfate, or any polycationic substance which is physiologically suitable, or a hybrid antibody directed against the envelope glycoprotein of the virus or the vector and the relevant target in the vessel wall or in the tissue perfused by the vessel to enhance the efficiency of infection by increasing adhesion of viral particles to the relevant target cells. The hybrid antibody directed against the envelope glycoprotein of the virus or the vector and the relevant target cell can be made by one of two methods. Antibodies directed against different epitopes can be chemically crosslinked (G. Jung, C. J. Honsik, R. A. Reisfeld, and H. J. Muller-Eberhard, *Proc. Natl. Acad. Sci. USA*, 83, 4479 (1986); U. D. Staerz, O. Kanagawa, and M. J. Bevan, *Nature*, 314, 628 (1985); and P. Perez, R. W. Hoffman, J. A. Titus, and D. M. Segal, *J. Exp. Med.*, 163, 166 (1986)) or biologically coupled using hybrid hybridomas (U. D. Staerz and M. J. Bevan, *Proc. Natl. Acad. Sci. USA*, 83, 1453 (1986); and C. Milstein and A. C. Cuello, *Nature*, 305, 537 (1983)). After incubation in the central space of the catheter for 0.2 to 2 hours or more, the medium is evacuated, gently washed with phosphate buffered saline, and circulation restored.

Using a different catheter design (see FIG. 2), a different protocol for instillation can also be used. This second approach involves the use of a single balloon means (2) catheter with multiple port means (3) which allow for high pressure delivery of the retrovirus into partially denuded arterial segments. The vessel surface is prepared as described above and defective vector is introduced using similar adhesive molecules. In this instance, the use of a high pressure delivery system serves to optimize the interaction of vectors with cells in adjacent vascular tissue.

The present invention also provides for the use of growth factors delivered locally by catheter or systemically to enhance the efficiency of infection. In addition to retroviral vectors, herpes virus, adenovirus, or other viral vectors are suitable vectors for the present technique.

It is also possible to transform cells within an organ or tissue. Direct transformation of organ or tissue cells may be accomplished by one of two methods. In a first method a high pressure transfection is used. The high pressure will cause the vector to migrate through the blood vessel walls into the surrounding tissue. In a second method, injection into a capillary bed, optionally after injury to allow leaking, gives rise to direct infection of the surrounding tissues.

The time required for the instillation of the vectors or cells will depend on the particular aspect of the invention being employed. Thus, for instilling cells or vectors in a blood vessel a suitable time would be from 0.01 to 12 hrs, preferably 0.1 to 6 hrs, most preferably 0.2 to 2 hrs. Alternatively for high pressure instillation of vectors or cells, shorter times might be preferred.

Obtaining the cells used in this invention

The term "genetic material" generally refers to DNA which codes for a protein. This term also encompasses RNA when used with an RNA virus or other vector based on RNA.

Transformation is the process by which cells have incorporated an exogenous gene by direct infection, transfection or other means of uptake.

The term "vector" is well understood and is synonymous with the often-used phrase "cloning vehicle". A vector is non-chromosomal double-stranded DNA comprising an intact replicon such that the vector is replicated when placed within a unicellular organism, for example by a process of transformation. Viral vectors include retroviruses, adenoviruses, herpesvirus, papovirus, or otherwise modified naturally occurring viruses. Vector also means a formulation of DNA with a chemical or substance which allows uptake by cells.

In another embodiment the present invention provides for inhibiting the expression of a gene. Four approaches may be utilized to accomplish this goal. These include the use of antisense agents, either synthetic oligonucleotides which are complementary to the mRNA (Maher III, L. J. and Dolnick, B. J. *Arch. Biochem. Biophys.*, 253, 214–220 (1987) and Zamecnik, P. C., et al., *Proc. Natl. Acad. Sci.*, 83, 4143–4146 (1986)), or the use of plasmids expressing the reverse complement of this gene (Izant, J. H. and Weintraub, H., *Science*, 229, 345–352, (1985); *Cell*, 36, 1077–1015 (1984)). In addition, catalytic RNAs, called ribozymes, can specifically degrade RNA sequences (Uhlenbeck, O. C., *Nature*, 328, 596–600 (1987), Haseloff, J. and Gerlach, W. L., *Nature*, 334, 585–591 (1988)). The third approach involves "intracellular immunization", where analogues of intracellular proteins can interfere specifically with their function (Friedman, A. D., Triezenberg, S. J. and McKnight, S. L., *Nature*, 335, 452–454 (1988)), described in detail below.

The first approaches may be used to specifically eliminate transcripts in cells. The loss of transcript may be confirmed by S1 nuclease analysis, and expression of binding protein determined using a functional assay. Single-stranded oligonucleotide analogues may be used to interfere with the processing or translation of the transcription factor mRNA. Briefly, synthetic oligonucleotides or thiol-derivative analogues (20–50 nucleotides) complementary to the coding strand of the target gene may be prepared. These antisense agents may be prepared against different regions of the mRNA. They are complementary to the 5' untranslated region, the translational initiation site and subsequent 20–50 base pairs, the central coding region, or the 3' untranslated region of the gene. The antisense agents may be incubated with cells transfected prior to activation. The efficacy of antisense competitors directed at different portions of the messenger RNA may be compared to determine whether specific regions may be more effective in preventing the expression of these genes.

RNA can also function in an autocatalytic fashion to cause autolysis or to specifically degrade complementary RNA sequences (Uhlenbeck, O. C., *Nature*, 328, 596–600 (1987), Haseloff, J. and Gerlach, W. L., *Nature*, 334, 585–591 (1988), and Hutchins, C. J., et al, *Nucleic Acids Res.*, 14, 3627–3640 (1986)). The requirements for a successful RNA cleavage include a hammerhead structure with conserved RNA sequence at the region flanking this structure. Regions adjacent to this cata-

lytic domain are made complementary to a specific RNA, thus targeting the ribozyme to specific cellular mRNAs. To inhibit the production of a specific target gene, the mRNA encoding this gene may be specifically degraded using ribozymes. Briefly, any GUG sequence within the RNA transcript can serve as a target for degradation by the ribozyme. These may be identified by DNA sequence analysis and GUG sites spanning the RNA transcript may be used for specific degradation. Sites in the 5' untranslated region, in the coding region, and in the 3' untranslated region may be targeted to determine whether one region is more efficient in degrading this transcript. Synthetic oligonucleotides encoding 20 base pairs of complementary sequence upstream of the GUG site, the hammerhead structure and ~20 base pairs of complementary sequence downstream of this site may be inserted at the relevant site in the cDNA. In this way, the ribozyme may be targeted to the same cellular compartment as the endogenous message. The ribozymes inserted downstream of specific enhancers, which give high level expression in specific cells may also be generated. These plasmids may be introduced into relevant target cells using electroporation and cotransfection with a neomycin resistant plasmid, pSV2-Neo or another selectable marker. The expression of these transcripts may be confirmed by Northern blot and S1 nuclease analysis. When confirmed, the expression of mRNA may be evaluated by S1 nuclease protection to determine whether expression of these transcripts reduces steady state levels of the target mRNA and the genes which it regulates. The level of protein may also be examined.

Genes may also be inhibited by preparing mutant transcripts lacking domains required for activation. Briefly, after the domain has been identified, a mutant form which is incapable of stimulating function is synthesized. This truncated gene product may be inserted downstream of the SV-40 enhancer in a plasmid containing the neomycin resistance gene (Mulligan, R. and Berg, P., *Science*, 209, 1422–1427 (1980) (in a separate transcription unit). This plasmid may be introduced into cells and selected using G418. The presence of the mutant form of this gene will be confirmed by S1 nuclease analysis and by immunoprecipitation. The function of the endogenous protein in these cells may be evaluated in two ways. First, the expression of the normal gene may be examined. Second, the known function of these proteins may be evaluated. In the event that this mutant intercellular interfering form is toxic to its host cell, it may be introduced on an inducible control element, such as metallothionein promoter. After the isolation of stable lines, cells may be incubated with Zn or Cd to express this gene. Its effect on host cells can then be evaluated.

Another approach to the inactivation of specific genes is to overexpress recombinant proteins which antagonize the expression or function of other activities. For example, if one wished to decrease expression of TPA (e.g., in a clinical setting of disseminate thrombolysis), one could overexpress plasminogen activator inhibitor.

Advances in biochemistry and molecular biology in recent years have led to the construction of "recombinant" vectors in which, for example, retroviruses and plasmids are made to contain exogenous RNA or DNA, respectively. In particular instances the recombinant vector can include heterologous RNA or DNA, by which is meant RNA or DNA that codes for a polypep-

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tide ordinarily not produced by the organism susceptible to transformation by the recombinant vector. The production of recombinant RNA and DNA vectors is well understood and need not be described in detail. However, a brief description of this process is included here for reference.

For example, a retrovirus or a plasmid vector can be cleaved to provide linear RNA or DNA having ligatable termini. These termini are bound to exogenous RNA or DNA having complementary like ligatable termini to provide a biologically functional recombinant RNA or DNA molecule having an intact replicon and a desired phenotypical property.

A variety of techniques are available for RNA and DNA recombination in which adjoining ends of separate RNA or DNA fragments are tailored to facilitate ligation.

The exogenous, i.e., donor, RNA or DNA used in the present invention is obtained from suitable cells. The vector is constructed using known techniques to obtain a transformed cell capable of in vivo expression of the therapeutic agent protein. The transformed cell is obtained by contacting a target cell with a RNA- or DNA-containing formulation permitting transfer and uptake of the RNA or DNA into the target cell. Such formulations include, for example, retroviruses, plasmids, liposomal formulations, or plasmids complexes with polycationic substances such as poly-L-lysine, DEAC-dextran and targeting ligands.

The present invention thus provides for the genetic alteration of cells as a method to transmit therapeutic or diagnostic agents to localized regions of the blood vessel for local or systemic purposes. The range of recombinant proteins which may be expressed in these cells is broad and varied. It includes gene transfer using vectors expressing such proteins as tPA for the treatment of thrombosis and restenosis, angiogenesis or growth factors for the purpose of revascularization, and vasoactive factors to alleviate vasoconstriction or vasospasm. This technique can also be extended to genetic treatment of inherited disorders, or acquired diseases, localized or systemic. The present invention may also be used to introduce normal cells to specific sites of cell loss, for example, to replace endothelium damaged during angioplasty or catheterization.

For example, in the treatment of ischemic diseases (thrombotic diseases), genetic material coding for tPA or modifications thereof, urokinase or streptokinase is used to transform the cells. In the treatment of ischemic organ (e.g., heart, kidney, bowel, liver, etc.) failure, genetic material coding for recollateralization agents, such as transforming growth factor α (TGF-α), transforming growth factor β (TGF-β) angiogenin, tumor necrosis factor α, tumor necrosis factor β, acidic fibroblast growth factor or basic fibroblast growth factor can be used. In the treatment of vasomotor diseases, genetic material coding for vasodilators or vasoconstrictors may be used. These include atrial natriuretic factor, platelet-derived growth factor or endothelin. In the treatment of diabetes, genetic material coding for insulin may be used.

The present invention can also be used in the treatment of malignancies by placing the transformed cells in proximity to the malignancy. In this application, genetic material coding for diphtheria toxin, pertussis toxin, or cholera toxin may be used.

In one of its embodiments, the present invention provides for the therapy of malignancy by either stimulat-

ing an immune response against tumor cells or inhibiting tumor cell growth or metastasis by genetic modification in vivo. This approach differs from previous methods in which tumor cells are propagated, modified, and selected in vitro.

In accordance with this embodiment, the present method is used to deliver a DNA sequence or an RNA sequence, including recombinant genes, to tumor cells in vivo with (1) retroviral or viral vectors as vehicles, (2) DNA or RNA/liposome complexes as vehicles, (3) chemical formulations containing the DNA or RNA sequence and coupled to a carrier molecule which facilitates delivery of the sequence to the targeted cells, or (4) by utilizing cell-mediated gene transfer to deliver genes to specific sites in vivo, e.g., by relying upon the use of vascular smooth muscle cells or endothelia cells which have been transduced in vitro as a vehicle to deliver the recombinant gene into the site of the tumor.

In an aspect of this embodiment, the present invention relies on the immune system to provide protection against cancer and play an important role as an adjuvant treatment for a malignancy. Immunotherapy has shown promise as an adjuvant approach to the treatment of malignancies. Both cytolytic T cells and lymphokines can facilitate tumor cell destruction, and strategies to enhance tumor regression by administration of cytokines or tumor infiltrating lymphocytes have shown efficacy in animal models and human trials. For example, it is known that lymphokine activated killer cells (LAK) and tumor infiltrating lymphocytes (TIL) can lyse neoplastic cells and produce partial or complete tumor rejection. Expression of cytokine genes in malignant cells has also enhanced tumor regression.

The present invention provides a novel gene transfer approach against tumors by the introduction of recombinant genes directly into tumor cells in vivo, where, by contrast, traditional gene transfer techniques have focused on modification of tumor cells in vitro followed by transfer of the modified cells. The prior art approaches are disadvantageous because they subject the cells to selection in different growth conditions from those which act in vivo, and because they also require that cell lines be established for each malignancy, thereby rendering adaptability to human disease considerably more difficult.

Genes which may be used with this embodiment include genes containing a DNA sequence (or the corresponding RNA sequence may be used) encoding an intracellular, secreted, or cell surface molecule which is exogenous to the patient and which (1) is immunogenic to the patient, (2) induces rejection, regression, or both, of the tumor, or (3) is toxic to the cells of the tumor.

The vectors containing the DNA sequence (or the corresponding RNA sequence) which may be used in accordance with the invention may be an eukaryotic expression vector containing the DNA or the RNA sequence of interest. Techniques for obtaining expression of exogenous DNA or RNA sequences in a host are known. See, for example, Korman et al, *Proc. Nat. Acad. Sci. (USA)*, (1987) 84:2150-2154, which is hereby incorporated by reference.

This vector, as noted above, may be administered to the patient in a retroviral or other viral vector (i.e., a viral vector) vehicle, a DNA or RNA/liposome complex, or by utilizing cell-mediated gene transfer. Further, the vector, when present in non-viral form, may be administered as a DNA or RNA sequence-containing chemical formulation coupled to a carrier molecule

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which facilitates delivery to the host cell. Such carrier molecule would include an antibody specific to the cells to which the vector is being delivered or a molecule capable of interacting with a receptor associated with the target cells.

Cell-mediated gene transfer may be used in accordance with the invention. In this mode, one relies upon the delivery of recombinant genes into living organisms by transfer of the genetic material into cells derived from the host and modification in cell culture, followed by the introduction of genetically altered cells into the host. An illustrative packaging cell line which may be used in accordance with this embodiment is described in Danos et al, *Proc. Natl. Acad. Sci. (USA)* (1988) 85:6460, which is hereby incorporated by reference.

The DNA or RNA sequence encoding the molecule used in accordance with the invention may be administered to the patient, which may be human or a non-human animal, either locally or systemically. The systemic administration is preferably carried out using the non-viral DNA or RNA chemical formulation coupled to a carrier molecule which facilitates delivery to the host cells. Any of the administrations may be performed by IV or IM injection or subcutaneous injection using any known means, or by the use of the catheter in accordance with the present invention.

The retroviral vector vehicles used in accordance with the present invention comprise a viral particle derived from a naturally-occurring retrovirus which has been genetically altered to render it replication defective and to express a recombinant gene of interest in accordance with the invention. Once the virus delivers its genetic material to a cell, it does not generate additional infectious virus but does introduce exogenous recombinant genes to the cell.

In other viral vectors, the virus particle used is derived from other naturally-occurring viruses which have been genetically altered to render them replication defective and to express recombinant genes. Such viral vectors may be derived from adenovirus, papillomavirus, herpesvirus, parvovirus, etc.

The sequences of the present invention may also be administered as DNA or RNA/liposome complex. Such complexes comprise a mixture of fat particles, lipids, which bind to genetic material, DNA or RNA, providing a hydrophobic coat, allowing genetic material to be delivered into cells. This formulation provides a non-viral vector for gene transfer. Liposomes used in accordance with the invention may comprise DOPE (dioleyl phosphatidyl ethanol amine), CUDMEDA (N-(5-cholestrum-3-β-ol 3-urethanyl)-N',N'-dimethylethylene diamine).

As noted above, other non-viral vectors may also be used in accordance with the present invention. These include chemical formulations of DNA or RNA coupled to a carrier molecule (e.g., an antibody or a receptor ligand) which facilitates delivery to host cells for the purpose of altering the biologic properties of the host cells. The term "chemical formulations" used herein refers to modifications of nucleic acids to allow coupling of the nucleic acid compounds to a protein or lipid, or derivative thereof, carrier molecule. Such carrier molecules include antibodies specific to the host cells or receptor ligands, i.e., molecules able to interact with receptors associated with the host cells.

The molecules which may be used in accordance with this invention, include the following: (1) genes encoding immune stimulants, such as Class I histocom-

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patibility genes, Class II histocompatibility genes, bacterial genes, including mycobacterial (PPD) genes and genes encoding heat shock proteins, viral glycoproteins encoding genes, including vesicular stomatitis virus G protein, influenza hemagglutinin, and herpes virus glycoprotein β, minor histocompatibility antigens, foreign proteins, such as lysozyme or bovine serum albumin, and oncogenes, including EIA, P53 (mutants) and tax; (2) immune and growth stimulants/inhibitors, including inducers of differentiation, such as stimulants, including interleukin-2 (IL-2) IL-4, 3, 6 or 8, inhibitors/inducers of differentiation, such as TNF-α or β, TGF-β (1, 2 or 3), IL-1, soluble growth factor receptors (PDGF, FGF receptors), recombinant antibodies to growth factors or receptors, analogs of growth factors (PDGF, FGF), interferons (α, β or γ) and adhesion molecules; or (3) toxins or negative selectable markers, including thymidine kinase, diphtheria toxin, pertussis toxin or drug-sensitive proteins.

The DNA/RNA sequence is preferably obtained from a source of the same species as the patient, but this is not absolutely required, and the present invention provides for the use of DNA sequences obtained from a source of a species different from the patient in accordance with this embodiment. A preferred embodiment of the present invention, genes encoding immune stimulants and toxins or negative selectable markers, corresponding to (1) and (3) above, are preferably selected from a species different than the species to which the patient belongs. For immune and growth stimulants/inhibitors, corresponding to (2) above, in accordance with another preferred embodiment of the invention, one preferably employs a gene obtained from a species which is the same as the species of the patient.

In the use of the present invention in the treatment of AIDS, genetic material coding for soluble CD4 or derivatives thereof may be used. In the treatment of genetic diseases, for example, growth hormone deficiency, genetic material coding for the needed substance, for example, human growth hormone, is used. All of these genetic materials are readily available to one skilled in this art.

In another embodiment, the present invention provides a kit for treating a disease in a patient which contains a catheter and a solution which contains either an enzyme or a mild detergent, in which the catheter is adapted for insertion into a blood vessel and contains a main catheter body having a balloon element adapted to be inserted into said vessel and expansible against the walls of the blood vessel so as to hold the main catheter body in place in the blood vessel, and means carried by the main catheter body for delivering a solution into the blood vessel, and the solution which contains the enzyme or mild detergent is a physiologically acceptable solution. The solution may contain a proteolytic enzyme, such as dispase, trypsin, collagenase, papain, pepsin, or chymotrypsin. In addition to proteolytic enzymes, liposes may be used. As a mild detergent, the solution may contain NP-40, Triton X100, deoxycholate, SDS or the like.

Alternatively, the kit may contain a physiological acceptable solution which contains an agent such as heparin, poly-L-lysine, polybrene, dextran sulfate, a polycationic material, or bivalent antibodies. This solution may also contain vectors or cells (normal or transformed). In yet another embodiment the kit may contain a catheter and both a solution which contains an enzyme or mild detergent and a solution which contains

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an agent such as heparin, poly-L-lysine, polybrene, dextran sulfate, a polycationic material or bivalent antibody and which may optionally contain vectors or cells.

The kit may contain a catheter with a single balloon and central distal perfusion port, together with acceptable solutions to allow introduction of cells in a specific organ or vectors into a capillary bed or cells in a specific organ or tissue perfused by this capillary bed.

Alternatively, the kit may contain a main catheter body which has two spaced balloon elements adapted to be inserted in a blood vessel with both being expansible against the walls of the blood vessel for providing a chamber in the blood vessel, and to hold the main catheter body in place. In this case, the means for delivering a solution into the chamber is situated in between the balloon elements. The kit may contain a catheter which possesses a plurality of port means for delivering the solution into the blood vessel.

Thus, the present invention represents a method for treating a disease in a patient by causing a cell attached onto the walls of a vessel or the cells of an organ perfused by this vessel in the patient to express an exogenous therapeutic agent protein, wherein the protein treats the disease or may be useful for diagnostic purposes. The present method may be used to treat diseases, such as an ischemic disease, a vasomotor disease, diabetes, a malignancy, AIDS or a genetic disease.

The present method may use exogenous therapeutic agent proteins, such as tPA and modifications thereof, urokinase, streptokinase, acidic fibroblast growth factor, basic fibroblast growth factor, tumor necrosis factor α, tumor necrosis factor β, transforming growth factor α, transforming growth factor β, atrial natriuretic factor, platelet-derived growth factor, endothelian, insulin, diphtheria toxin, pertussis toxin, cholera toxin, soluble CD4 and derivatives thereof, and growth hormone to treat diseases.

The present method may also use exogenous proteins of diagnostic value. For example, a marker protein, such as β-galactosidase, may be used to monitor cell migration.

It is preferred, that the cells caused to express the exogenous therapeutic agent protein be endothelial cells.

Other features of the present invention will become apparent in the course of the following descriptions of exemplary embodiments which are given for illustration of the invention and are not intended to be limiting thereof.

The data reported below demonstrate the feasibility of endothelial cell transfer and gene transplantation; that endothelial cells may be stably implanted in situ on the arterial wall by catheterization and express a recombinant marker protein, β-galactosidase, in vivo.

Because atherogenesis in swine has similarities to humans, an inbred pig strain, the Yucatan minipig (Charles River Laboratories, Inc., Wilmington, Mass.), was chosen as an animal model (1). A primary endothelial cell line was established from the internal jugular vein of an 8 month-old female minipig. The endothelial cell identity of this line was confirmed in that the cells exhibited growth characteristics and morphology typical of porcine endothelium in tissue culture. Endothelial cells also express receptors for the acetylated form of low density lipoprotein (AcLDL), in contrast to fibroblasts and other mesenchymal cells (2). When analyzed for ACLDL receptor expression, greater than 99% of

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the cultured cells contained this receptor, as judged by fluorescent ACLDL uptake.

Two independent β-galactosidase-expressing endothelial lines were isolated following infection with a murine amphotropic β-galactosidase-transducing retroviral vector (BAG), which is replication-defective and contains both β-galactosidase and neomycin resistance genes (3). Cells containing this vector were selected for their ability to grow in the presence of G-418. Greater than 90% of selected cells synthesized β-galactosidase by histochemical staining. The endothelial nature of these genetically altered cells was also confirmed by analysis of fluorescent ACLDL uptake. Infection by BAG retrovirus was further verified by Southern blot analysis which revealed the presence of intact proviral DNA at approximately one copy per genome.

Endothelial cells derived from this inbred strain, being syngeneic, were applicable for study in more than one minipig, and were tested in nine different experimental subjects. Under general anesthesia, the femoral and iliac arteries were exposed, and a catheter was introduced into the vessel (FIG. 1). Intimal tissues of the arterial wall were denuded mechanically by forceful passage of a partially inflated balloon catheter within the vessel. The artery was rinsed with heparinized saline and incubated with the neutral protease, dispase (50 U/ml), which removed any remaining luminal endothelial cells. Residual enzyme was rapidly inactivated by α2 globulin in plasma upon deflating the catheter balloons and allowing blood to flow through the vessel segment. The cultured endothelial cells which expressed β-galactosidase were introduced using a specially designed arterial catheter (USCI, Billerica, Mass.) that contained two balloons and a central instillation port (FIG. 1).

When these balloons were inflated, a protected space was created within the artery into which cells were instilled through the central port 3 (FIG. 1). These endothelial cells, which expressed β-galactosidase, were allowed to incubate for 30 minutes to facilitate their attachment to the denuded vessel. The catheter was then removed, the arterial branch ligated, and the incision closed.

Segments of the artery inoculated with β-galactosidase-expressing endothelium were removed 2 to 4 weeks later. Gross examination of the arterial specimen after staining using the X-gal chromogen showed multiple areas of blue coloration, compared to an artery seeded with uninfected endothelium, indicative of β-galactosidase activity. Light microscopy documented β-galactosidase staining primarily in endothelial cells of the intima in experimentally seeded vessels.

In contrast, no evidence of similar staining was observed in control segments which had received endothelial cells containing no β-galactosidase. β-Galactosidase staining was occasionally evident in deeper intimal tissues, suggesting entrapment or migration of seeded endothelium within the previously injured vessel wall. Local thrombosis was observed in the first two experimental subjects. This complication was minimized in subsequent studies by administering acetylsalicylic acid prior to the endothelial cell transfer procedure and use of heparin anticoagulation at the time of inoculation. In instances of thrombus formation, β-galactosidase staining was seen in endothelial cells extending from the vessel wall to the surface of the thrombus.

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A major concern of gene transplantation in vivo relates to the production of replication-competent retrovirus from genetically engineered cells. In these tests, this potential problem has been minimized through the use of a replication defective retrovirus. No helper virus was detectable among these lines after 20 passages in vitro. Although defective viruses were used because of their high rate of infectivity and their stable integration into the host cell genome (4), this approach to gene transfer is adaptable to other viral vectors.

A second concern involves the longevity of expression of recombinant genes in vivo. Endothelial cell expression of β-galactosidase appeared constant in vessels examined up to six weeks after introduction into the blood vessel in the present study.

These tests have demonstrated that genetically-altered endothelial cells can be introduced into the vascular wall of the Yucatan minipig by arterial catheterization. Thus, the present method can be used for the localized biochemical treatment of vascular disease using genetically-altered endothelium as a vector.

A major complication of current interventions for vascular disease, such as balloon angioplasty or insertion of a graft into a diseased vessel, is disruption of the atherosclerotic plaque and thrombus formation at sites of local tissue trauma (5). In part, this is mediated by endothelial cell injury. (6). The present data show that genetically-altered endothelial cells can be introduced at the time of intervention to minimize local thrombosis.

This technique can also be used in other ischemic settings, including unstable angina or myocardial infarction. For instance, antithrombotic effects can be achieved by introducing cells expressing genes for tissue plasminogen activator or urokinase. This technology is also useful for the treatment of chronic tissue ischemia. For example, elaboration of angiogenic or growth factors (7) to stimulate the formation of collateral vessels to severely ischemic tissue, such as the myocardium. Finally, somatic gene replacement for systemic inherited diseases is feasible using modifications of this endothelial cell gene transfer technique.

Another aspect of the present invention relates a method for modulating the immune system of an animal by in vivo transformation of cells of the animal with a recombinant gene. The transformation may be carried out either in a non-site-specific or systemic manner or a site-specific manner. If the transformation is carried out in a systemic fashion or at sites other than those which confer specificity on the immune system, such as the thymus, then the immune system will be modulated to result in the animal being sensitized to the molecule for which the recombinant gene encodes. Alternatively, if the transformation is carried out in a site-specific manner and is localized to a site which determines the specificity of the immune system, e.g., the thymus, the immune system will be modulated to result in the animal being tolerized to the molecule encoded by the recombinant gene.

By the term sensitized, it is meant that the immune system exhibits a stronger response to the molecule encoded by the DNA after in vivo transformation as compared to before transformation. By the term tolerized, it is meant that the immune system displays a reduced response to the molecule encoded by the recombinant gene after transformation as compared to before transformation. Thus, one may modulate an immune system to provide either a resistance or a tolerance to the molecule encoded by the DNA.

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Examples of molecules for which it may be desirable to provide a resistance to include: cell surface molecules, such as tumor antigens (carcinoembryonic antigen), protozoan antigens (pneumocystis), viral antigens (HIV gp120 and gp160, H. influenza antigen, and hepatitis B surface antigen), Lyme disease antigen, Bacterial antigens, and transplantation antigens (Class I or II), ras or other oncogenes, including erb-A or neu; cytoplasmic proteins, such as the raf oncogene, src oncogene, and abl oncogene; nuclear proteins, such as E1A oncogene, mutant p53 oncogene, tat, tax, rev, vpu, vpx, hepatitis core antigen, EBNA and viral genes; and secreted proteins, such as endotoxin, cholera toxin, TNF, and osteoclast activating factor.

Examples of molecules for which it may be desirable to provide a resistance to include: cell surface molecules, such as growth factor receptors, insulin receptors, thyroid hormone receptors, transplantation antigens (class I or II), blood group antigens, and LDL receptor; cytoplasmic proteins, such as cytochrome P450, galactosyl transferase, dystrophin, neomycin resistance gene, and bacterial heat shock protein; nuclear proteins, such as retinoblastoma and transdominant rev; and secreted proteins, such as growth hormone for dwarfs, insulin for diabetics, and adenosine deaminase.

It is to be understood that the nucleic acid, DNA, RNA, or derivative thereof, in the recombinant gene may be of any suitable origin. That is the nucleic acid may be isolated from a naturally occurring source or may be of synthetic origin.

The recombinant gene may be introduced in the cells of the animal using any conventional vector. Such vectors include viral vectors, cationic lipids complexed to DNA or RNA (DNA or RNA/liposomes) and DNA or RNA complexes with polycations, such as DEAE, dextran, and polybrene.

As noted above the recombinant gene can be introduced into cells in a site-specific manner to confer resistance to the molecule encoded by the recombinant gene. Suitable sites include, e.g., endothelial cells or reticuloendothelial cells in the vasculature or any specific tissue or organ. The form of the preparation containing the vector and recombinant gene used in the transformation will depend on the specific tissue to be transformed. Suitable preparations for transforming endothelial cells are described elsewhere in this specification. In addition, preparations suitable for oral or other means of administration (e.g., endoscopic) may be used to provide mucosal resistance. Such preparation could include detergents, gelatins, capsules or other delivery vehicles to protect against degradation and enhance delivery to the mucosal surface, in addition to the vector and gene.

Alternatively, the recombinant gene may be introduced in a site specific fashion to a site which determines the specificity of the immune system. The thymus is such a site (see: A. M. Posselt et al, Science, vol. 249, p. 1292 (1990)). Thus, by introducing a recombinant gene site-specifically into the thymus, the immune system may be modulated to result in a tolerance to the molecule encoded by the gene. In this way, transplant rejection may be suppressed. The same preparations and techniques used to site-specifically transform tumors described above may be used to introduce the recombinant gene into the thymus. Specifically, the transformation preparation may be injected directed into the thymus or tumor or into the vascular supply of the thymus or tumor.

The present method may be practiced on any animal, such as chickens or mammals such as cows, horses, cats, dogs, monkeys, lemurs or humans.

When the recombinant gene is introduced using a liposome, it is preferred to first determine in vitro the optimal values for the DNA: lipid ratios and the absolute concentrations of DNA and lipid as a function of cell death and transformation efficiency for the particular type of cell to be transformed and to use these values in the in vivo transformation. The in vitro determination of these values can be easily carried out using the techniques described in the Experimental Section of this specification.

Another aspect of the present invention relates to a kit for the in vivo systemic introduction of a recombinant gene into cells of an animal. Such a kit would include approximately the optimal amount of a carrier, such as a lipid, and nucleic acid, and/or a means of delivery, e.g., an endoscope or a syringe. The kit may also contain instructions for the administration of the transforming preparation. The carrier and nucleic acid may be freeze dried and may be packaged separately or premixed. The kit may also contain a solution to optimally reconstitute the complexes of the carrier and the nucleic acid, which provide for efficient delivery to cells in vivo. Such a solution may contain one or more ingredients, such as buffers, sugars, salts, proteins, and detergents.

Having generally described the invention, a further understanding can be obtained by reference to certain specific examples which are provided herein for purposes of illustration only and are not intended to be limiting unless otherwise specified.

Experimental section

A. Analysis of AcLDL receptor expression in normal and β-galactosidase-transduced porcine endothelial cells.

Endothelial cell cultures derived from the Yucatan minipig, two sublines infected with BAG retrovirus or 3T3 fibroblast controls were analyzed for expression of AcLDL receptor using fluorescent labelled AcLDL.

Endothelial cells were derived from external jugular veins using the neutral protease dispase (8). Excised vein segments were filled with dispase (50 U/ml in Hanks' balanced salt solution) and incubated at 30° C. for 20 minutes. Endothelium obtained by this means was maintained in medium 199 (GIBCO, Grand Island, N.Y.) supplemented with fetal calf serum (10%), 50 μg/ml endothelial cell growth supplement (ECGS) and heparin (100 μg/ml). These cells were infected with BAG retrovirus, and selected for resistance to G-418. Cell cultures were incubated with (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbacyanine perchlorate) (DiI) AcLDL (Biomedical Technologies, Stoughton, Mass.) (10 μg/ml) for 4–6 hrs. at 37° C., followed by three rinses with phosphate-buffered saline containing 0.5% glutaraldehyde. Cells were visualized by phase contrast and fluorescent microscopy.

B. Method of introduction of endothelial cells by catheterization.

A double balloon catheter was used for instillation of endothelial cells. The catheter has a proximal and distal balloon, each 6 mm in length and 5 mm in width, with a 20 mm length between the balloons. The central section of the catheter has a 2 mm pore connected to an instillation port. Proximal and distal balloon inflation isolates a central space, allowing for instillation of in-

fected cells through the port into a discrete segment of the vessel. For a schematic representation of cell introduction by catheter, see FIGS. 1 and 2.

Animal care was carried out in accordance with "Principles of Laboratory Animal Care" and "Guide for the Care and Use of Laboratory Animals" (NIH publication No. 80-23, Revised 1978). Female Yucatan minipigs (80–100 kg) were anesthetized with pentobarbital (20 mg/kg), intubated, and mechanically ventilated. These subjects underwent sterile surgical exposure of the iliac and femoral arteries. The distal femoral artery was punctured, and the double-balloon catheter was advanced by guidewire into the iliac artery. The external iliac artery was identified; the proximal balloon was partially inflated and passed proximally and distally so as to mechanically denude the endothelium. The catheter was then positioned with the central space located in the region of denuded endothelium, and both balloons were inflated. The denuded segment was irrigated with heparinized saline, and residual adherent cells were removed by instillation of dispase (20 U/ml) for 10 min. The denuded vessel was further irrigated with a heparin solution and the BAG-infected endothelial cells were instilled for 30 min. The balloon catheter was subsequently removed, and antegrade blood flow was restored. The vessel segments were excised 2 to 4 weeks later. A portion of the artery was placed in 0.5% glutaraldehyde for five minutes and stored in phosphate-buffered saline, and another portion was mounted in a paraffin block for sectioning. The presence of retroviral expressed β-galactosidase was determined by a standard histochemical technique (19).

C. Analysis of endothelial cells in vitro and in vivo.

β-Galactosidase activity was documented by histochemical staining in (A) primary endothelial cells from the Yucatan minipig, (B) a subline derived by infection with the BAG retroviral vector, (C) a segment of normal control artery, (D) a segment of artery instilled with endothelium infected with the BAG retroviral vector, (E) microscopic cross-section of normal control artery, and (F) microscopic cross-section of artery instilled with endothelium infected with the BAG retroviral vector.

Endothelial cells in tissue culture were fixed in 0.5% glutaraldehyde prior to histochemical staining. The enzymatic activity of the E. coli β-galactosidase protein was used to identify infected endothelial cells in vitro and in vivo. The β-galactosidase transducing Mo-MuLV vector (2), (BAG) was kindly provided by Dr. Constance Cepko. This vector used the wild type Mo-MuLV LTR as a promoter for the β-galactosidase gene. The simian virus 40 (SV-40) early promoter linked to the Tn5 neomycin resistance gene provides resistance to the drug G-418 and is inserted downstream of the β-galactosidase gene, providing a marker to select for retrovirus-containing, β-galactosidase expressing cells. This defective retrovirus was prepared from fibroblast ψ am cells (3,10), and maintained in Dulbecco's modified Eagle's medium (DMEM) and 10% calf serum. Cells were passaged twice weekly following trypsinization. The supernatant, with titers of 10^4–10^5/ml G-418 resistant colonies, was added to endothelial cells at two-thirds confluence and incubated for 12 hours in DMEM with 10% calf serum at 37° C. in 5% CO_2 in the presence of 8 μg/ml of polybrene. Viral supernatants were removed, and cells maintained in medium 199 with 10% fetal calf serum, ECGS (50 μg/ml), and endothelial cell conditioned medium (20%)

for an additional 24 to 48 hours prior to selection in G-418 (0.7 pg/ml of a 50% racemic mixture). G-418 resistant cells were isolated and analyzed for β-galactosidase expression using a standard histochemical stain (9). Cells stably expressing the β-galactosidase enzyme were maintained in continuous culture for use as needed. Frozen aliquots were stored in liquid nitrogen.

D. Immunotherapy of Malignancy by In Vivo Gene Transfer.

A retroviral vector which the H-2K^S gene was prepared. CT26 cells were infected with this vector in vitro, selected for G418 resistance, and analyzed by fluorescence activated cell sorting (FACS). Transduced CT26 cells showed a higher mean fluorescence intensity than uninfected CT26 cells or CT26 infected with different retroviral vectors. When 10^6 CT26 cells which express H-2K^S were injected subcutaneously into BALB/c mice (H-2^d) sensitized to this antigen, no tumors were observed over an 8-week period in contrast to the unmodified CT26 (H-2^d) tumor line which routinely formed tumors at this dose. The immune response to H-2K^S could therefore provide protection against CT26 cells bearing this antigen. When CT26 H-2K^S and CT26 were co-inoculated, however, tumor growth was observed, suggesting that. H-2K^S conferred senstivity only to modified cells.

To determine whether protective effects could be achieved by introduction of H-2K^S in growing CT26 tumors, the recombinant H-2K^S reporter or a β-galactosidase gene was introduced into tumors either with a DNA/liposome or a retroviral vector. Tumor capsules (0.5–1 cm diameter) were exposed surgically and multiple needle injections (2–10) delivered to the parenchyma. With β-galactosidase reporter plasmids, recombinant gene expression could be readily detected after intra-tumor injection of DNA/liposome or retroviral vectors.

In mice which received intra-tumor injections of the H-2K^S DNA/liposome complex or H-2K^S retroviral vector, the recombinant DNA was detected by PCR in the tumor and occasionally in other tissues. When found in the other organs, no evidence of inflammation or ogran toxicity was detected pathologically. An immune response to the recombinant H-2K^S protein was evident in these animals, however. Lymphocytes derived from the H-2K^S, but not β-galactosidase transduced tumors, demonstrated a cytolytic response to H-2K^S whether delivered by retroviral vectors or liposomes. More importantly, lymphocytes derived from the H-2K^S, but not β-galactosidase transduced animals, recognized and lysed unmodified CT26 cells, indicating that this stimulation induced immune reactivity against genetically unmodified tumor cells.

To assess the protective effect of the immune response against H-2K^S, tumor growth in vivo was quantitated. When animals received no prior sensitization to H-2K^S, one of four tumors transduced with H-2K^S showed attenuation of tumor growth which was not complete. In contrast, no anti-tumor effect was seen in unmodified (n=4) or β-galactosidase transduced controls (n=4). Because these tumors were large at the time of initial injection and continued to grow as the primary immune response was generated, an attempt was made to optimize the anti-tumor response by pre-immunization of mice with irradiated CT26 H-2K^S tumor cells, and by earlier and/or more frequent injections of vector. Tumors were transduced on days 12 and 32 by intra-tumor injuection of H-2K^S or β-galactosi-

dase DNA/liposome vectors. Treatment with the H-2K^S liposome complex improved survival and attenuated tumor growth, in contrast to β-galactosidase transduced tumors where there was no difference in growth rate compared to the uninjected controls. Complete tumor regression was acheived in two mice by increasing the number of injections and by delivery of H-2K^S into tumors at an earlier stage. This treatment was protective, since control animals showed continued tumor growth and did not survive beyond 35 days.

E. Modulation of the Immune System.

The response to injection of cationic lipids and plasmids was determined after injection intravenously into BALB/c mice (6–12 weeks). In the first experiments, a gene encoding the H-2K^S molecule was introduced by tail vein injection. Two to four weeks later, spleen cells were harvested and analyzed for their ability to mediate a cytolytic T cell response. When these cells were tested using ^51Cr target cells (CT26 cells expressing the H-2K^S gene), significant cytolysis was observed which was not seen in animals injected with the cointrol vector, β-galactosidase (see FIG. 3). Up to 25% of target cells were lysed at effector: target ratios of 25:1.

In addition to this specific cytolytic T cell response, serologic or antibody responses to genes encoded by expression vector plasmids have been examined. When a plasmnid encoding the gp160 molecule of HIV is injected, an antibody response is elicited in treated mice. In contrast to control animals injected with cationic lipids containing β-galactosidase, mice injected with cationic lipids with gp 160 plasmid showed an antibody response to the gp160 and gp120 form of this molecule by Western blot analysis (See FIG. 4). These results demonstrate that systemic administration of cationic lipid/DNA complexes can be used successfully to induce cell-mediated and antibody-mediated immunity against foreign pathogens.

F. Determination of Optimal Transfection Conditions.

(1) Plasmid Construction

A plasmid containing the E. coli lacZ gene under the control of the Rous Sarcoma Virus LTS (RSV-β-gal) (Norton and Coffin, Mol. Cell. Biol., 5(2), 281–290, 1985) was used for transfection of porcine primary endothelial and HeLa cells. In addition, a plasmid containing the lacZ gene under the control of preproendothelin-1 5'-flanking DNA (−1410 to +83) (Wilson et al., Mol. Cell. Biol., 10(9), 4854–4862, 1990) was used for transfection of endothelial cells. For in vivo toxicity analysis, the RSV-β-gal plasmid, and a plasmid derived from the PLJ vector containing the cDNA encoding an H-2K^S mouse MHC class I gene were used.

(2) Cell Culture, Transfection Analysis, and Toxicity in Vitro

Primary endothelial cells, derived from the Yucatan minipig (YPE cells), were incubated with medium 199 (M199) supplemented with 10% FBS, 2 mM 1-glutamine, 50 U/ml penicillin, and 5 μg/ml streptomycin. HeLa cells were maintained in Dulbeccos Modified Eagles Medium (DMEM) supplemented with 5% FBS, 2 mM 1-glutamine, 50 U/ml penicillin and 5 μg/ml streptomycin. The DNA liposome mixture was prepared with lipid concentrations of DOPE/DC-Chol between 2.5 and 25 μM added to 0.2 ml of serum-free media or Ringer's lactate solution in polystyrene tubes. After mixing gently, the solution was allowed to stand at room temperature for 15–20 minutes. For transfection analysis, cells were grown in 60 mm tissue culture

dishes at 75% confluency or greater. Cells were washed twice with serum-free media or lactated Ringers solution and then placed in 0.5 mls of the same media. The DNA liposome solution (0.2 ml) was then added slowly to the cells, with gentle mixing, with a final volume of 0.7 ml. This resulted in DNA concentrations between 0.7 and 7 μg/ml (13–130 nM), and lipid concentrations of 7–70 μM. Transfection was allowed to proceed for 1–5 hours, after which the cells were placed in media supplemented as decribed above. At 24–48 hours after transfection the enzymatic activity of the *E. coli* β-galactosidase protein was used to identify transfected cells by staining with the X-gal chromagen. Toxicity in vitro was assessed by cytopathic effect or trypan blue exclusion.

(3) Animal Studies

For intravenous injections, the DNA/liposomes were prepared as described for the in vitro transfection studies in 0.2 ml of serum-free M199 or lactated-Ringers solution. After 15–20 min of incubation, the mixture was diluted to 0.7 ml and 0.1 to 0.2 ml of this dilution was then injected immediately into the tail vein of adult, female BALB/c mice. Blood was collected before injection and 9–11 days following injection, and serum chemistries were examined. At ~2–3 weeks following injection, the liver, kidney, lung, heart, and brain were extracted for histologic and PCR DNA amplification analysis as described previously. Intratumor injection of CT26 cells (Fearon et al., *Cell*, 60, 397–403, 1990) and analysis were also performed according to the previous protocols.

(4) Results

The optimal conditions for transfection and toxicity of DNA/liposomes were initially determined in vitro. To obtain maximal transfection without toxicity in vitro, the ratio of DNA to cationic lipid, the absolute concentration of DNA or lipids, and the conditions for mixture of DNA and cationic lipids were studied. The cationic lipid preparation was a formulation of two compounds, which include dioleoyl phosphatidylethanolamine (DOPE) and cholesten-3-β-ol 3-urethanyl-N',N' dimethylethylene diamine (DC-chol). The transfection efficiencies of this reagent were equal to or greater than those of Lipofectin ® (BRL) in several cell lines in vitro. Endothelial cells, which are typically difficult to transfect, and HeLa cells, which can be transfected easily using a variety of techniques, were examined by transfection in vitro.

To determine the optimal conditions for transfection of endothelial cells, the lipid was initially used at different concentrations while the DNA concentration was held constant. Maximal transfection efficiency was seen using 0.7 μg/ml DNA (13 nM) and 21 μM of DO-PE/DCOChol lipid, with a sharp decline in the number of transfected cells with higher or lower lipid concentrations. Next, the DNA concentration was altered as the lipid concentration remained constant. This analysis revealed a similar sensitivity to DNA concentration, with the number of transfected cells decreasing significantly with increments of DNA concentration as low as 0.4 μg/ml. These results indicate that the ratio of DNA to lipid is important for maximum transfection efficiency, and that the absolute concentration of each component is also important in determining the efficiency of transfection. An increase in DNA and lipid concentration beyond the optimal concentration of 0.7 μg/ml DNA (13 nM) and 21 μM of DOPE/DC-Chol reduced the number of viable cells and did not increase

the transfection efficiency of the remaining viable cells. Lipid concentrations greater than 35 μM reduced the number of viable cells by 50% compared to the untransfected control, whereas the optimal concentration of 0.7 μg/ml DNA (13 nM) ande 21 μM of lipid had no effect on cell viability after 5 hours of incubation.

To compare the optimal concentrations of transfection in a different cell type, transfections were performed on HeLa cells. In this case, a slightly different optimal ratio of DNA and lipid were observed. Peak transfection efficiencies were obtained at the same lipid concentration as endothelial cells (21 μg/ml) but varied less with small differences in DNA concentrations. DNA concentrations of 1.4–4.2 μg/ml were equally effective. Again, when the ratio of DNA to lipid was maintained but the concentration of each was decreased three-fold, very few cells were transfected, illustrating that both the ratio of DNA to lipid and the absolute concentration of each component are important in maximizing the number of transfected cells. If HeLa cells were transfected at >80% confluence or greater, there was no toxicity using up to 35 μM of lipid. When cells were transfected at a lower saturation density, however, cell viability was reduced dramatically with as little as 7 μM of lipid compared to the untransfected control cells. These results demonstrate that the optimal conditions for transfection and toxicity may differ somewhat depending on the cell line.

Another variable in the preparation of liposomes was the composition of the solution used to generate complexes of the cationic lipids with DNA. Among several media solutions analyzed, no substantial difference was noted in transfection efficiency or toxicity with M199, McCoys, OptiMEM, or RPMI media. A significant improvement in transfection efficiency was observed, however, using standard Ringers lactate. The number of transfected cells increased more than 3-fold compared to the serum-free medium, although prolonged incubation (≧2 hours) resulted in a loss of cell viability in some cell types.

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Obviously, numerous modifications and variations of the present invention are possible in light of the above-teachings. It is therefore to be understood that, within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.

We claim:

1. A kit for treating a disease in a patient in need thereof, comprising a catheter and a physiologically acceptable solution, wherein:

(i) said catheter is adapted for insertion into a blood vessel and comprises a main catheter body having a balloon element, adapted to be inserted in said blood vessel and being expansible against the walls of said vessel so as to hold said main catheter body in place, and means carried by said main catheter body for delivering said solution into said blood vessel;

(ii) said physiologically acceptable solution comprises DNA and at least one member selected from the group consisting of heparin, poly-L-lysine, polybrene, dextransulfate, a polycationic material, and bivalent antibodies.

2. The kit of claim 1, wherein said physiologically acceptable solution further comprises a growth factor.

3. A kit for treating a disease in a patient in need thereof, comprising

(i) a catheter adapted for insertion into a blood vessel, comprising a main catheter body having a balloon element adapted to being inserted into said vessel and expansible against the walls of the said vessels so as to hold said main catheter body in place in said vessel and a means carried by said main catheter body for delivering a physiologically acceptable solution into said blood vessel;

(ii) said physiologically acceptable solution which may contain an enzyme, mild detergent or lipid; and

(iii) a means for causing a cell attached onto the walls of a vessel or in an organ or tissue in said patient to express an exogenous therapeutic agent protein, comprising a formulation adapted for delivery by said catheter for the transfer and uptake of RNA or DNA into said cell attached onto the walls of a vessel or in an organ or tissue in said patient.

4. The kit according to claim 3, wherein said DNA is antisense DNA.

5. The kit of claim 3, wherein said solution contains, as said enzyme, at least one member selected from the group consisting of dispase, trypsin, collagenase, papain, pepsin, chymotrypsin, and lipases.

6. The kit of claim 3, wherein said solution contains at least one member selected from the group consisting of Nonidet P-40, Triton X100, deoxycholate, and sodium dodecyl sulfate.

7. The kit of claim 3, wherein said main catheter body comprises two spaced balloon elements, adapted to be inserted in a blood vessel and both being expansible against the walls of the blood vessel, for providing a chamber in said blood vessel and so as to hold said main catheter body in place, and whereas said means for delivering a physiologically acceptable solution into said chamber is situated in between said balloon elements.

8. The kit of claim 3, wherein said means for delivering said solution into said blood vessel comprises a plurality of pore means.

9. The kit of claim 3, wherein said formulation comprises a retrovirus, a plasmid, a liposomal formulation, or a plasmid complex with a polycationic substance.

10. The kit of claim 3, wherein said formulation is a liposomal formulation.

* * * * *

EVIDENCE APPENDIX

ITEM NO. 17

Declaration of Dr. G. Robert Meger cited by Appellant as an
Exhibit in the Amendment filed February 15, 2001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: James P. Elia   )
)
SERIAL NO.: 09/064,000   )   EXAMINER: Nicholas D. Lucchesi
)
FILED: April 21, 1998   )
)   GROUP ART UNIT: 3732
FOR: METHOD AND APPARATUS   )
      FOR INSTALLATION OF   )
      DENTAL IMPLANT   )

DECLARATION OF G. ROBERT MEGER, M.D.

I G. Robert Meger declare as follows:

1.   I have offices at 3333 East Camelback Road, Phoenix, Arizona 85018.

2.   My Curriculum Vitae is attached hereto as Exhibit A.

3.   I have read and understood the disclosures at column 14, lines 4-61 and column
        21, lines 1-26 of United States Patent Number 5,397,235 (hereinafter " '235
        patent") entitled "Method for Installation of Dental Implant," and granted to
        James P. Elia on March 14, 1995. A copy of such disclosures is attached hereto
        as Exhibit B. I understand that the same disclosures are contained in above patent
        Application Serial No. 09/064,000.

4.   I note that the disclosures mentioned in above Paragraph 3 relate to a method for
        forming a bud and resulting soft tissue. Such methods involve placing a growth
        factor at a desired site of a body with use of techniques including resorbable and
        non-resorbable carriers, gels, time-release capsules, and granules. In addition, the
        growth factor may be placed in the body orally, systemically, by injection,

I

through the respiratory tract, by making an incision in the body and then inserting the growth factor. I note further that the growth factor and/or carrier may be activated by tissue pH, enzymes, ultrasound, electricity, heat, or in vivo chemicals.

5.   The materials included in Exhibit C of this Declaration illustrate that the techniques set forth in above Paragraph 4 were well known to those skilled in the medical arts prior to July 2, 1993. It is my opinion that one skilled in the medical arts armed with such knowledge would have been able to practice the invention(s) described at column 14, lines 4-61 and column 21, lines 1-26 of the '235 patent without need for resorting to undue experimentation.

6.   Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 2/13/01   /s/ Robert Mejia
                                          G. Robert Mejia

EXHIBIT A

CURRICULUM VITAE

Exhibit A

CURRICULUM VITAE

G. Robert Meger, M.D.

Birthplace:	Cedar Rapids, Iowa
Birth date:	July 19, 1964
Address:	3333 E Camelback Rd Suite 140 Phoenix, AZ 85018
Phone:	(602) 957-6000
Fax:	(602) 957-6349
E Mail:	Megermd@home.com
Board Certifications:	American Board of Plastic Surgery American Board of Surgery
Memberships:	American Society of Plastic Surgeons Maricopa County Society of Plastic Surgeons American Medical Association
License:	Board of Medical Examiners State of Arizona #23447
Residency:	Plastic Surgery Medical College of Georgia Augusta, Georgia

General Surgery
Phoenix Integrated Surgical Residency
Good Samaritan Regional Medical Center
Phoenix, Arizona

Surgical Internship
Maricopa Medical Center
Phoenix, Arizona

Education: Doctor of Medicine
Creighton University
Omaha, Nebraska

Bachelor of Science in Chemistry
University of Notre Dame
Notre Dame, Indiana

EXHIBIT
B

DISCLOSURES

Growth factors can be utilized to induce the growth of "hard tissue" or bone and "soft tissues" like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basic)(FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 (OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors, and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound, by electricity, by heat, by selected in vivo chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor can also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such a small incision comprises minor surgery which can often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

In another embodiment of the invention, genetically produced living material is used to form an implant in the bone of a patient. The DNA structure of a patient is analyzed from a sample of blood or other material extracted from a patient and a biocompatible tooth bud 122 (FIG. 3) is produced. The bud 122 is placed in an opening 123 in the alveolar bone and packing material is placed around or on top of the bud 122. The size of opening 123 can vary as desired. The packing around bud 122 can comprise HAC 124, hydroxyapatite, blood, growth factors, or any other desirable packing material. The bud 122 grows into a full grown tooth in the same manner that tooth buds which are in the jaws of children beneath baby teeth grow into full sized teeth. Instead of bud 122, a quantity of genetically produced living material which causes bud 122 to form in the alveolar bone can be placed at a desired position in the alveolar bone such that bud 122 forms and grows into a full sized tooth. Instead of forming an opening 123, a needle or other means can be used to simply inject the genetically produced living material into a selected location in the alveolar bone. As would be appreciated by those skilled in the art, genetically produced materials can be inserted in the body to cause the body to grow, reproduce, and replace leg bone, facial bone, and any other desired soft and hard tissue in the body.

EXHIBIT C

EXHIBIT C
SUMMARY OF MATERIALS

TECHNIQUES OF INTRODUCING
AND ACTIVATION OF GROWTH FACTORS

EXH. NO.	MATERIAL AND DATE	TECHNIQUE
C-1	J Periodontal, November 1991, "Effects of platelet-derived growth fctor/insulin-like growth factor-1 combination on bone regeneration around titanium dental implants". Lynch S.E., et. al.	Gel carrier
C-2	Nature, November 28, 1991, "Electrically erodible polymer gel for controlled release of drugs". Kwon, I.C., et. al.	Possibility of multiple chemical release stimuli of gel for controlled release
C-3	Acta Orthop Scand, October 1991, "Dose-dependent stimulation of bone induction by basic fibroblast growth in rats". Aspenberg P., et. al.	Gel carrier
C-4	Natl. Acad. Sci., November 1992, "Heat shock induces the release of fibroblast growth factor 1 from NIH 3T3 cells". Jackson A., et. al.	Heat activation of growth factor
C-5	Transplant, 1992, "Cell transplantation for myocardial repair: an experimental approach". Marelli D., et. al.	Heart injection
C-6	Lasers Sur. Med., 1989, "Macrophage responsiveness to light therapy". Young, S.	Light activation
C-7	J Surg. Res., May 1989, "Attachment of peptide growth factors to implantable collagen". Stompro B.E., et. al.	Absorbable carrier
C-8	Clin. Orthop., February 1991, "Bone morphogenesis of rabbit bone morphogenetic protein-bound hydroxyapatite-fibrin composite". Sato T., et. al.	Non-absorbable carrier
C-9	Arch Surg., June 1989, "Angiotropin treatment prevents flap necrosis and enhances dermal regeneration in rabbits". Hockel M., Burke J.F.	Injection

EXH. NO.	MATERIAL AND DATE	TECHNIQUE
C-10	JAMA, October, 1991, "Tissue transformation into bone in vivo. A potential practical application". Khouri R.K., et. al.	Injection
C-11	Radiology, December 1986, "An experimental evaluation of microcapsules for arterial chemoembolilzation". Bechtel W., et. al.	Intra Arterial capsule delivery
C-12	Atherosclerosis, February 1989, "Histopathologic examination of material from angioplasty balloon catheters used in vivo in human coronary arteries". Sprecher D.L., et. al.	Coronary heart catheter
C-13	Int. J Cancer, May 1989, "Acidic Cellular Environments: activation of latent TGF-beta and sensitization of cellular responses to TGF-beta and EGF". Dullien P., et. al.	pH activation
C-14	Atherosclerosis, April 1990, "Endothelial cell stimulation of smooth muscle glycosamino-glycan sythesis can be accounted for by transforming growth factor beta activity". Merrilees M.J., Scott L.	Heat activation
C-15	Ultrasound Med Biol, 1990, "Macrophage responsiveness to therapeutic ultrasound". Young S.R., Dyson M.	Ultrasound activation
C-16	Am J Physiol, September 1989, "Mitogenic signals for thrombin in mesangial cells: regulation of phosspholipase C and PDGF genes". Schultz P.J., et. al.	Enzyme activation
C-17	J Burn Cure Rehabil, July-August, 1991, "Weak direct current accelerates split-thickness healing on tangentially excised second-degree burns". Chu C.S., et. al.	Electrical activation

2

List Contains 1 Item.
Current Search Formulation: +LYNCH SE; + 1991 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Effects of the platelet-derived growth factor/insulin-like growth factor-I combination on bone regeneration around titanium dental implants. Results of a pilot study in beagle dogs.
ARTICLE SOURCE: J Periodontol (United States), Nov 1991, 62(11) p710-6
AUTHOR(S): Lynch SE; Buser D; Hernandez RA; Weber HP; Stich H; Fox CH; Williams RC
AUTHOR'S ADDRESS: Department of Periodontology, Harvard School of Dental Medicine, Boston, MA.
MAJOR SUBJECT HEADING(S): Bone Regeneration [drug effects]; Dental Implantation, Endosseous; Dental Implants; Insulin-Like Growth Factor I [therapeutic use]; Mandible [surgery]; Platelet-Derived Growth Factor [therapeutic use]; Titanium
MINOR SUBJECT HEADING(S): Analysis of Variance; Dogs; Drug Combinations; Gels; Insulin-Like Growth Factor I [administration & dosage]; Mandible [pathology] [physiopathology]; Methylcellulose; Pilot Projects; Placebos; Platelet-Derived Growth Factor [administration & dosage]; Recombinant Proteins; Wound Healing
INDEXING CHECK TAG(S): Animal; Female; Support, Non-U.S. Gov't; Support, U.S. Gov't, P.H.S.
PUBLICATION TYPE: JOURNAL ARTICLE

ABSTRACT: The purpose of this study was to evaluate the early wound healing events of bone around press-fit titanium implants inserted with and without the concurrent application of a combination of platelet-derived growth factor (PDGF) and insulin-like growth factor (IGF-I). Nine months prior to implant placement all mandibular premolar teeth were extracted in 8 beagle dogs. Subsequently, 40 specially manufactured titanium implants with 2 transverse holes in the apical section were press fit into precise recipient sites in the dogs' mandibles. The dogs were sacrificed at 7 and 21 days following implant placement yielding 12 PDGF-B/IGF-I treated and 8 control (placebo gel or non-treated) implants for each observation period. Coded undecalcified sections were analyzed for: 1) percentage of implant surface in contact with new bone; 2) percentage of peri-implant space filled with new bone; and 3) percentage of implant hole filled with new bone. An analysis of variance was used to determine significant differences among the treatment groups. At 7 days, the percentage of bone fill in the peri-implant spaces and the percentage of implant surface in contact with new bone were both significantly increased in PDGF-B/IGF-I treated sites (P less than 0.01 for both groups). There was less than 1.5% fill of the implant holes in both treated and control sites (no significant differences). At 21 days the percentage of bone fill in the peri-implant spaces was significantly increased in the PDGF-B/IGF-I treated sites (P less than 0.01).(ABSTRACT TRUNCATED AT 250 WORDS).
MEDLINE INDEXING DATE: 199204
ISSN: 0022-3492
LANGUAGE: English
UNIQUE NLM IDENTIFIER: 92092155
CAS REGISTRY/EC NUMBER(S): 0 (Dental Implants); 0 (Drug Combinations); 0 (Gels); 0 (Placebos); 0 (Platelet-Derived Growth Factor); 0 (Recombinant Proteins); 67763-96-6 (Insulin-Like Growth Factor I); 7440-32-6 (Titanium); 9004-67-5 (Methylcellulose)
GRANT ID NUMBER: 5T32 DE07010-DE-NIDR; K16 DE 0027501-DE-NIDR

EXHIBIT C-1

List Contains 1 Item.
Current Search Formulation: "gel delivery"

This Document Selected From: 1986 - 1995 SurgAnLine® [1996 Edition]

ARTICLE TITLE: Electrically erodible polymer gel for controlled release of drugs.
ARTICLE SOURCE: Nature (England), Nov 28 1991, 354(6351) p291-3
AUTHOR(S): Kwon IC; Bae YH; Kim SW
AUTHOR'S ADDRESS: Center for Controlled Chemical Delivery, University of Utah, Salt Lake City 84108.
MAJOR SUBJECT HEADING(S): Delayed-Action Preparations
MINOR SUBJECT HEADING(S): Acrylic Resins [chemistry]; Electric Stimulation; Hydrogen-Ion Concentration; Insulin [administration & dosage]; Oxazoles [chemistry]; Polymers [chemistry]; Polymethacrylic Acids [chemistry]; Solubility
PUBLICATION TYPE: JOURNAL ARTICLE

ABSTRACT: New controlled drug-delivery systems are being explored to overcome the disadvantages of conventional dosage forms. For example, stimulated drug-delivery has been used to overcome the tolerance problems that occur with a constant delivery rate, to mimic the physiological pattern of hormonal concentration and to supply drugs on demand. Stimuli-sensitive polymers, which are potentially useful for pulsed drug delivery, experience changes in either their structure or their chemical properties in response to changes in environmental conditions. Environmental stimuli include temperature, pH, light (ultraviolet or visible), electric field or certain chemicals. Volume changes of stimuli-sensitive gel networks are particularly responsive to external stimuli, but swelling is slow to occur. As well as being useful in the controlled release of drugs, such systems also provide insight into intermolecular interactions. Here we report on a novel polymeric system, which rapidly changes from a solid state to solution in response to small electric currents, by disintegration of the solid polymer complex into two water-soluble polymers. We show that the modulated release of insulin, and by extension other macromolecules, can be achieved with this polymeric system.

MEDLINE INDEXING DATE: 9203
ISSN: 0028-0836
LANGUAGE: English
UNIQUE NLM IDENTIFIER: 92065953
CAS REGISTRY/EC NUMBER(S): 0 (Acrylic Resins); 0 (Delayed-Action Preparations); 0 (Oxazoles); 0 (Polymers); 0 (Polymethacrylic Acids); 11061-68-0 (Insulin); 25087-26-7 (polymethacrylic acid); 25805-17-8 (polyethyloxazoline); 9003-01-4 (carbopol 940)

EXHIBIT C-2

List Contains 1 Item.
Current Search Formulation: +ASPENBERG P; + 1991 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Dose-dependent stimulation of bone induction by basic fibroblast growth factor in rats.
ARTICLE SOURCE: Acta Orthop Scand (Denmark), Oct 1991, 62(5) p481-4
AUTHOR(S): Aspenberg P; Thorngren KG; Lohmander LS
AUTHOR'S ADDRESS: Lund University Hospital Department of Orthopedics, Sweden.
MAJOR SUBJECT HEADING(S): Bone Matrix [transplantation]; Fibroblast Growth Factor, Basic [pharmacology]; Osteogenesis [drug effects]
MINOR SUBJECT HEADING(S): Abdominal Muscles [surgery]; Bone Matrix [chemistry]; Calcium [analysis]; Dose-Response Relationship, Drug; Fibroblast Growth Factor, Basic [administration & dosage]; Rats, Inbred Strains; Rats
INDEXING CHECK TAG(S): Animal; Female; Support, Non-U.S. Gov't
PUBLICATION TYPE: JOURNAL ARTICLE

ABSTRACT: Implantation of demineralized bone matrix in rodents elicits a series of cellular events leading to the formation of new bone inside and adjacent to the implant. This process is believed to be initiated by an inductive protein present in bone matrix, and local growth factors may further regulate the process. We have previously shown that local application of recombinant human basic fibroblast growth factor (bFGF) in a carboxymethyl cellulose gel to demineralized bone matrix implants increases the bone yield as measured by calcium content 3 weeks after implantation in rats. We now report that this increase was seen at 3 and 4 weeks, but not earlier or later. Further, the stimulatory effect was seen with doses from 3 to 75 ng per implant. A dose of 0.6 or 380 ng did not increase the bone yield, and 1,900 ng had a marked inhibitory effect. This narrow dosage optimum may reflect the complex actions of the growth factor.

MEDLINE INDEXING DATE: 199202
ISSN: 0001-6470
LANGUAGE: English
UNIQUE NLM IDENTIFIER: 92057648
CAS REGISTRY/EC NUMBER(S): 0 (Fibroblast Growth Factor, Basic); 7440-70-2 (Calcium)

EXHIBIT C-3

i

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Details of Search | Return to Results | Return to Search Screen

Selected full citations from 114 MEDLINE records

Related Articles | External Links

TITLE:	Heat shock induces the release of fibroblast growth factor 1 from NIH 3T3 cells.
AUTHORS:	Jackson A; Friedman S; Zhan X; Engleka KA; Forough R; Maciag T
AUTHOR AFFILIATION:	Department of Molecular Biology, Jerome H. Holland Laboratory for the Biomedical Sciences, American Red Cross, Rockville, MD 20855.
SOURCE:	Proc Natl Acad Sci U S A 1992 Nov 15;89(22):10691-5
CITATION IDS:	PMID: 1279690 UI: 93066309

ABSTRACT:

Fibroblast growth factor 1 (FGF-1) is a potent angiogenic and neurotrophic factor whose structure lacks a classical signal sequence for secretion. Although the initiation of these biological activities involves the interaction between FGF-1 and cell surface receptors, the mechanism responsible for the regulation of FGF-1 secretion is unknown. We report that murine NIH 3T3 cells transfected with a synthetic gene encoding FGF-1 secrete FGF-1 into their conditioned medium in response to heat shock. The form of FGF-1 released by NIH 3T3 cells in response to increased temperature (42 degrees C, 2 hr) in vitro is not biologically active and does not associate with either heparin or the extracellular NIH 3T3 monolayer matrix. However, it was possible to derive biologically active FGF-1 from the conditioned medium of heat-shocked NIH 3T3 cell transfectants by ammonium sulfate fractionation. The form of FGF-1 exposed by ammonium sulfate fractionation is similar in size to cytosolic FGF-1 and can bind and be eluted from immobilized heparin similarly to the recombinant human FGF-1 polypeptide. Further, the release of FGF-1 by NIH 3T3 cell transfectants in response to heat shock is reduced significantly by both actinomycin D and cycloheximide. These data indicate that increased temperature may upregulate the expression of a factor responsible for the secretion of FGF-1 as a biologically

EXHIBIT C-4

inactive complex that requires an activation step to exhibit the biological activity of the extracellular polypeptide mitogen.

MAIN MESH HEADINGS:	Fibroblast Growth Factor, Acidic/*biosynthesis *Heat
ADDITIONAL MESH HEADINGS:	Animal Cell Division Culture Media, Conditioned Cycloheximide/pharmacology Cytosol/metabolism Dactinomycin/pharmacology DNA/biosynthesis Fibroblast Growth Factor, Acidic/genetics Fibroblast Growth Factor, Acidic/pharmacology Fibroblast Growth Factor, Acidic/secretion Genes, Synthetic Immunoblotting Kinetics Mice Recombinant Proteins/pharmacology Support, U.S. Gov't, P.H.S. Thymidine/metabolism Transfection Tritium 3T3 Cells 1992/11 1992/15 00:00
PUBLICATION TYPES:	JOURNAL ARTICLE
CAS REGISTRY NUMBERS:	0 (Culture Media, Conditioned) 0 (Recombinant Proteins) 10028-17-8 (Tritium) 104781-85-3 (Fibroblast Growth Factor, Acidic) 50-76-0 (Dactinomycin) 50-89-5 (Thymidine) 66-81-9 (Cycloheximide) 9007-49-2 (DNA)
LANGUAGES:	Eng
GRANT/CONTRACT ID:	HL32348/HL/NHLBI HL44336/HL/NHLBI

List Contains 1 Item.
Current Search Formulation: +MARELLI D

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Cell transplantation for myocardial repair: an experimental approach.
ARTICLE SOURCE: Cell Transplant (United States), 1992, 1(6) p383-90
AUTHOR(S): Marelli D; Desrosiers C; el-Alfy M; Kao RL; Chiu RC
AUTHOR'S ADDRESS: Department of Surgery, McGill University, Montreal, Quebec, Canada.
MAJOR SUBJECT HEADING(S): Muscles [transplantation]; Myocardial Diseases [surgery]; Myocardium [pathology]; Transplantation, Heterotopic
MINOR SUBJECT HEADING(S): Cells, Cultured; Dogs; Freezing; Muscles [cytology] [physiology]; Myocardial Diseases [pathology]; Regeneration; Tissue Culture [methods]; Transplantation, Autologous; Transplantation, Heterotopic [methods] [physiology]
INDEXING CHECK TAG(S): Animal; Support, Non-U.S. Gov't
PUBLICATION TYPE: JOURNAL ARTICLE
ABSTRACT: Myocardium lacks the ability to regenerate following injury. This is in contrast to skeletal muscle (SKM), in which capacity for tissue repair is attributed to the presence of satellite cells. It was hypothesized that SKM satellite cells multiplied in vitro could be used to repair injured heart muscle. Fourteen dogs underwent explantation of the anterior tibialis muscle. Satellite cells were multiplied in vitro and their nuclei were labeled with tritiated thymidine 24 h prior to implantation. The same dogs were then subjected successfully to a myocardial injury by the application of a cryoprobe. The cells were suspended in serum-free growth medium and autotransplanted within the damaged muscle. Medium without cells was injected into an adjacent site to serve as a control. Endpoints comprised histology using standard stains as well as Masson trichrome (specific for connective tissue), and radioautography. In five dogs, satellite cell isolation, culture, and implantation were technically satisfactory. In three implanted dogs, specimens were taken within 6-8 wk. There were persistence of the implantation channels in the experimental sites when compared to the controls. Macroscopically, muscle tissue completely surrounded by scar tissue could be seen. Masson trichrome staining showed homogeneous scar in the control site, but not in the test site where a patch of muscle fibres containing intercalated discs (characteristic of myocardial tissue) was observed. In two other dogs, specimens were taken at 14 wk postimplantation. Muscle tissue could not be found. These preliminary results could be consistent with the hypothesis that SKM satellite cells can form neo-myocardium within an appropriate environment. Our specimens failed to demonstrate the presence of myocyte nuclei.(ABSTRACT TRUNCATED AT 250 WORDS).
MEDLINE INDEXING DATE: 199407
ISSN: 0963-6897
LANGUAGE: English
UNIQUE NLM IDENTIFIER: 94199205

EXHIBIT C-5

List Contains 1 Item.
Current Search Formulation: +YOUNG S; + 1989 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Macrophage responsiveness to light therapy.
ARTICLE SOURCE: Lasers Surg Med (United States), 1989, 9(5) p497-505
AUTHOR(S): Young S; Bolton P; Dyson M; Harvey W; Diamantopoulos C
AUTHOR'S ADDRESS: Anatomy Department, United Medical School, Guy's Hospital, London, England.
MAJOR SUBJECT HEADING(S): Growth Substances [physiology]; Lasers [therapeutic use]; Macrophages [radiation effects]; Wound Healing [radiation effects]
MINOR SUBJECT HEADING(S): Cell Division [radiation effects]; Cell Line; Cells, Cultured; Fibroblasts [cytology] [radiation effects]; Growth Substances [secretion]; Kidney [cytology]; Macrophages [cytology] [secretion]; Mice
INDEXING CHECK TAG(S): Animal; Comparative Study; In Vitro; Support, Non-U.S. Gov't
PUBLICATION TYPE: JOURNAL ARTICLE

ABSTRACT: Macrophages are a source of many important mediators of wound repair. It was the purpose of this study to see if light could stimulate the release of these mediators. In this study an established macrophage-like cell line (U-937) was used. The cells were exposed in culture to the following wavelengths of light: 660 nm, 820 nm, 870 nm, and 880 nm. The 820-nm source was coherent and polarised, and the others were non-coherent. Twelve hours after exposure the macrophage supernatant was removed and placed on 3T3 fibroblast cultures. Fibroblast proliferation was assessed over a 5-day period. The results showed that 660-nm, 820-nm, and 870-nm wavelengths encouraged the macrophages to release factors that stimulated fibroblast proliferation above the control levels, whereas the 880-nm wavelength either inhibited the release of these factors or encouraged the release of some inhibitory factors of fibroblast proliferation. These results suggest that light at certain wavelengths may be a useful therapeutic agent by providing a means of either stimulating or inhibiting fibroblast proliferation where necessary. At certain wavelengths coherence is not essential.
MEDLINE INDEXING DATE: 199002
ISSN: 0196-8092
LANGUAGE: English
UNIQUE NLM IDENTIFIER: 90042969
CAS REGISTRY/EC NUMBER(S): 0 (Growth Substances)

EXHIBIT C-6

Knowledge Finder®: Retrieved Documents   Page 1 Fri Jan 12 04:43:14 2001

List Contains 1 Item.
Current Search Formulation: +STOMPRO BE; + <u>1989</u> - All Articles

This Document Selected From: <u>1989</u> - 1998 SurgAnLine® [1999 Edition]

<u>ARTICLE TITLE:</u> Attachment of peptide growth factors to implantable collagen.
<u>ARTICLE SOURCE:</u> J Surg Res (United States), May <u>1989</u>, 46(5) p413-21
<u>AUTHOR(S):</u> <u>Stompro BE</u>; Hansbrough JF; Boyce ST
<u>AUTHOR'S ADDRESS:</u> Department of Surgery, University of California, San Diego Medical Center 92103.
<u>MAJOR SUBJECT HEADING(S):</u> Collagen; Epidermal Growth Factor-Urogastrone; Epidermis [cytology]; Growth Substances; Heparin; Keratin
<u>MINOR SUBJECT HEADING(S):</u> Cell Division; Cells, Cultured; Drug Combinations; Epidermal Growth Factor-Urogastrone [pharmacology]; Growth Substances [pharmacology]; Heparin [pharmacology]; Wound Healing
<u>INDEXING CHECK TAG(S):</u> Support, U.S. Gov't, P.H.S.
<u>PUBLICATION TYPE:</u> JOURNAL ARTICLE

<u>ABSTRACT:</u> Ingrowth of fibrovascular tissue from the woundbed into collagen-based dermal substitutes and survival of cultured epithelium after transplantation may be enhanced by attachment of heparin binding growth factor 2 (HBGF2) and epidermal growth factor (EGF) to collagen. Biotinylation of collagen and the growth factors allows immobilization of HBGF2 and EGF by high affinity binding of tetravalent avidin. Biotinylated HBGF2 and EGF (B-GF) were exposed to complexes of biotinylated collagen (B-COL)-avidin (A) and detected with peroxidase-labeled avidin (AP) followed by chromagen formation on nitrocellulose paper. Binding of biotinylated HBGF2 and EGF was specific (*, P less than 0.05), proportional to the concentration of biotinylated collagen, and resistant to ionic (NaCl) displacement. Data are expressed as mean percentages of maximum binding +/- SEMs: (table; see text) Growth response of cultured human epidermal keratinocytes to HBGF2 (population doubling time, PDT = 0.70 population doublings (PD)/day) confirmed the retention of mitogenic activity after biotinylation (PDT = 0.80 PD/day). Specific binding of biotinylated HBGF2, EGF, or other biologically active molecules (antibiotics, NSAIDs) to implantable collagen may provide a mechanism for positive therapeutic modulation of wound healing, including repair of full-thickness skin wounds with cultured cell-collagen composite grafts.
<u>MEDLINE INDEXING DATE:</u> 198908
<u>ISSN:</u> 0022-4804
<u>LANGUAGE:</u> English
<u>UNIQUE NLM IDENTIFIER:</u> 89237142
<u>CAS REGISTRY/EC NUMBER(S):</u> 0 (Drug Combinations); 0 (Fibroblast Growth Factor, Basic); 0 (Growth Substances); 62229-50-9 (Epidermal Growth Factor-Urogastrone); 68238-35-7 (Keratin); 9005-49-6 (Heparin); 9007-34-5 (Collagen)
<u>GRANT ID NUMBER:</u> GM35068-GM-NIGMS

EXHIBIT C-7

List Contains 1 Item.
Current Search Formulation: +SATO T; + <u>1991</u> - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Bone morphogenesis of rabbit bone morphogenetic protein-bound hydroxyapatite-fibrin composite.
ARTICLE SOURCE: Clin Orthop (United States), Feb <u>1991</u>, (263) p254-62
AUTHOR(S): Sato T; Kawamura M; Sato K; Iwata H; Miura T
AUTHOR'S ADDRESS: Department of Orthopaedic Surgery, Nagoya University School of Medicine, Japan.
MAJOR SUBJECT HEADING(S): Composite Resins [therapeutic use]; Fibrin [therapeutic use]; Growth Substances [therapeutic use]; Osteogenesis [drug effects]; Proteins [therapeutic use]
MINOR SUBJECT HEADING(S): Bone and Bones [drug effects]; Composite Resins [pharmacology]; Fibrin [pharmacology]; Growth Substances [pharmacology]; Hydroxyapatites [therapeutic use]; Proteins [pharmacology]; Rabbits
INDEXING CHECK TAG(S): Animal
PUBLICATION TYPE: JOURNAL ARTICLE

ABSTRACT: Hydroxyapatite (HAP) and fibrin have been implanted in patients and observed to be well tolerated in orthotopic sites. This is a report on a composite of HAP, fibrin, and rabbit bone morphogenetic protein and insoluble noncollagenous protein (BMP-iNCP). Drill holes in the femoral condyles of rabbits were packed with granulated HAP (200 mg), fibrin (0.3 ml), BMP-iNCP (5 mg), or various combinations of the two. The fibrin consisted mainly of sterilized human fibrinogen and thrombin, and BMP-iNCP was prepared from demineralized rabbit cortical bone. New bone formation was observed at one, two, four, and eight weeks after implantation. The BMP-iNCP augmented new bone formation in rabbit femoral condyles. Fibrin made the composite easier to manipulate and did not inhibit osteogenesis at any period. The composites of HAP with BMP-iNCP and of HAP with BMP-iNCP and fibrin produced higher yields of new bone than fibrin alone or HAP alone.
MEDLINE INDEXING DATE: 199105
ISSN: 0009-921X
LANGUAGE: English
UNIQUE NLM IDENTIFIER: 91130138
CAS REGISTRY/EC NUMBER(S): 0 (Bone Morphogenetic Proteins); 0 (Composite Resins); 0 (Growth Substances); 0 (Hydroxyapatites); 1306-06-5 (Durapatite); 9001-31-4 (Fibrin)

EXHIBIT C-8

List Contains 1 Item.
Current Search Formulation: +HOCKEL M; + 1989 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Angiotropin treatment prevents flap necrosis and enhances dermal regeneration in rabbits.
ARTICLE SOURCE: Arch Surg (United States), Jun 1989, 124(6) p693-8
AUTHOR(S): Hockel M; Burke JF
AUTHOR'S ADDRESS: Universitatsfrauenklinik Mainz, West Germany.
MAJOR SUBJECT HEADING(S): Angiogenesis Factor [pharmacology]; Growth Substances [pharmacology]; Necrosis [prevention & control]; Skin [pathology]; Surgical Flaps
MINOR SUBJECT HEADING(S): Angiogenesis Factor [administration & dosage]; Graft Survival; Injections, Intradermal; Rabbits; Skin [blood supply]; Wound Healing
INDEXING CHECK TAG(S): Animal; Female; Male
PUBLICATION TYPE: JOURNAL ARTICLE

ABSTRACT: Angiotropin is a potent angiogenesis factor isolated from the serum-free media of cultured, lectin-activated peripheral monocytes. In vitro, the purified substance stimulates migration, phenotypic differentiation, and tube formation, but not proliferation of capillary endothelial cells. When injected intradermally, angiotropin induces, in dose-dependent fashion, angiogenesis associated with skin hyperplasia. We have developed a flap model with insufficient blood supply and a model for contraction-free defect healing in rabbit skin. We show that (1) local pretreatment with angiotropin can prevent flap necrosis and (2) dermal regeneration after wounding can be augmented by angiotropin. From these results, we conclude that angiotropin might be of use as an adjuvant to healing in surgery.

MEDLINE INDEXING DATE: 198909
ISSN: 0004-0010
LANGUAGE: English
UNIQUE NLM IDENTIFIER: 89272615
CAS REGISTRY/EC NUMBER(S): 0 (Angiogenesis Factor); 0 (Growth Substances)

EXHIBIT C-9

List Contains 1 Item.
Current Search Formulation: +KHOURI RK; + 1991 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Tissue transformation into bone in vivo. A potential practical application.
ARTICLE SOURCE: JAMA (United States), Oct 9 1991, 266(14) p1953-5
AUTHOR(S): Khouri RK; Koudsi B; Reddi H
AUTHOR'S ADDRESS: Department of Surgery, Washington University School of Medicine, St Louis, Mo. 63110.
MAJOR SUBJECT HEADING(S): Bone and Bones [physiopathology]; Glycoproteins [administration & dosage]; Growth Substances [administration & dosage]; Muscles [transplantation]; Osteogenesis; Proteins [administration & dosage]
MINOR SUBJECT HEADING(S): Bone Matrix; Bone and Bones [surgery]; Injections; Osteogenesis [drug effects]; Rats, Inbred Lew; Rats; Surgical Flaps; Tissue Transplantation [methods]
INDEXING CHECK TAG(S): Animal; Support, Non-U.S. Gov't; Support, U.S. Gov't, P.H.S.
PUBLICATION TYPE: JOURNAL ARTICLE

ABSTRACT: The transformation of mesenchymal tissue, such as muscle, into cartilage and bone can be induced by the recently purified osteoinductive factor, osteogenin, and by its parent substratum, demineralized bone matrix. We investigated the possibility of transforming readily available muscle flaps into vascularized bone grafts of various shapes that could be used as skeletal replacement parts. In a rat experimental model, thigh adductor muscle island flaps were placed inside bivalved silicone rubber molds. Prior to closure of the mold, 18 flaps were injected with osteogenin and coated with demineralized bone matrix. Five flaps served as controls and were injected with the vehicle only, and not coated with demineralized bone matrix. The molds were implanted subcutaneously in the rats' flanks and reopened 10 days later. The control flaps consisted of intact muscle without any evidence of tissue transformation, whereas the flaps treated with osteogenin and demineralized bone matrix were entirely transformed into cancellous bone that matched the exact shape of the mold. Using tissue transformation, we were able to generate in vivo, autogenous, well-perfused bones in the shapes of femoral heads and mandibles.

MEDLINE INDEXING DATE: 199112
ISSN: 0098-7484
LANGUAGE: English
UNIQUE NLM IDENTIFIER: 91374707
CAS REGISTRY/EC NUMBER(S): 0 (osteogenin); 0 (osteoinductive factor); 0 (Glycoproteins); 0 (Growth Substances)
GRANT ID NUMBER: 22-3335 44901A

EXHIBIT C-10

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Details of Search | Return to Results | Return to Search Screen

Selected full citations from 1 MEDLINE records

Related Articles | External Links

TITLE: An experimental evaluation of microcapsules for arterial chemoembolization.

AUTHORS: Bechtel W; Wright KC; Wallace S; Mosier B; Mosier D; Mir S; Kudo S

SOURCE: Radiology 1986 Dec;161(3):601-4

CITATION IDS: PMID: 2947261 UI: 87068344

ABSTRACT: Microcapsules, 106 micron (range, 50-350 micron), of different capsular materials (monoglyceride, monodiglyceride, natural wax, cellulose polymer, or lactic acid polymer) with and without floxuridine (2'-deoxy-5-fluorouridine, FUDR) were intraarterially injected into dog kidneys. The drug-release characteristics of the microcapsules, as determined by analysis of renal and systemic venous blood samples over a 6-hour period, were uniphasic or multiphasic depending on the capsular material. Histologic changes of varying degrees were noted in all kidneys embolized except for those subjected to capsules of the cellulose polymer. The most striking changes were produced by the lactide polymer capsules. The potential applications of microencapsuled chemotherapeutic agents in intraarterial transcatheter treatment of cancer are discussed.

MAIN MESH HEADINGS: Antineoplastic Agents/*administration & dosage
*Embolization, Therapeutic

ADDITIONAL MESH HEADINGS: Animal
Antineoplastic Agents/blood
Capsules
Combined Modality Therapy
Dogs
Floxuridine/administration & dosage
Floxuridine/blood
Renal Artery
Support, Non-U.S. Gov't
Support, U.S. Gov't, P.H.S.
1986/12

EXHIBIT C-11

Knowledge Finder®: Retrieved Documents   Page 1 Fri Jan 12 05:26:50 2001

List Contains 1 Item.
Current Search Formulation: +SPRECHER DL; + 1989 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Histopathologic examination of material from angioplasty balloon catheters used in vivo in human coronary arteries.
ARTICLE SOURCE: Atherosclerosis (Netherlands), Feb 1989, 75(2-3) p237-44
AUTHOR(S): Sprecher DL; Mikat EM; Stack R; Sutherland K; Schneider J; Bashore T; Hackel DB
AUTHOR'S ADDRESS: University of Cincinnati Medical Center, Department of Pathology, OH 45267-0529.
MAJOR SUBJECT HEADING(S): Angioplasty, Balloon; Arteriosclerosis [pathology]; Atherosclerosis [pathology]; Coronary Vessels [pathology]; Specimen Handling [methods]
MINOR SUBJECT HEADING(S): Adult; Aged; Angina Pectoris [therapy]; Coronary Vessels [cytology]; Middle Age; Myocardial Infarction [therapy]
INDEXING CHECK TAG(S): Female; Human; Male; Support, U.S. Gov't, P.H.S.
PUBLICATION TYPE: JOURNAL ARTICLE

ABSTRACT: Reports on vascular pathology post-PTCA in both human and animal coronary vessels have revealed medial and intimal cracks and tears, thrombus formation, platelet accumulation, and loss of endothelial cells. The extent and type of damage can currently be assessed in vivo at the macro level by means of coronary artery angiography. However, this technique cannot define vessel wall characteristics at the cellular level. Our hypothesis is that vessel wall material may adhere to the balloon and thus provide a source for coronary artery cytological investigation in vivo. Ten balloon catheters were evaluated to discern any material which was dislodged from the coronary artery and which remained attached to the balloon catheter or guide wire. Our results indicate that angioplasty catheter balloons frequently have adherent collagen, endothelial cells, organized thrombus, and plaque with obvious cholesterol clefts, that can be retrieved and examined histologically. We conclude that material is often dislodged from the plaque during PTCA. In addition, plaque material removed by the balloon catheter offers an unusual opportunity to analyze the morphologic characteristics of cells from the human coronary artery in vivo.

MEDLINE INDEXING DATE: 198908
ISSN: 0021-9150
LANGUAGE: English
UNIQUE NLM IDENTIFIER: 89228141
GRANT ID NUMBER: HLB 17670

EXHIBIT C-12

List Contains 1 Item.
Current Search Formulation: +JULLIEN P; + 1989 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Acidic cellular environments: activation of latent TGF-beta and sensitization of cellular responses to TGF-beta and EGF.
ARTICLE SOURCE: Int J Cancer (United States), May 15 1989, 43(5) p886-91
AUTHOR(S): Jullien P; Berg TM; Lawrence DA
AUTHOR'S ADDRESS: Unite 532 CNRS, Institut Curie-Biologie, Orsay, France.
MAJOR SUBJECT HEADING(S): Cell Transformation, Neoplastic; Epidermal Growth Factor-Urogastrone [pharmacology]; Transforming Growth Factors [biosynthesis]
MINOR SUBJECT HEADING(S): Agar; Blood; Cell Division [drug effects]; Cell Line; Culture Media; Hydrogen-Ion Concentration; Lactates [pharmacology]; Mice; Transforming Growth Factors [pharmacology]
INDEXING CHECK TAG(S): Animal; Support, Non-U.S. Gov't
PUBLICATION TYPE: JOURNAL ARTICLE

ABSTRACT: Transient (about 2 hr) acidification to approx. pH 5.0 of agar-gelled overlayers containing untransformed NRK-49F or KiMSV-transformed NRK-49F cells in the presence of fetal calf serum or crude 49F-cell conditioned medium, as sources of latent TGF-beta, elicited EGF-dependent colony formation of 49F cells and inhibited spontaneous growth of transformed cells. Pure, active TGF-beta (porcine, type I) had the same effects on these respective cell types, suggesting that the above results were due to activation of latent TGF-beta in the transiently acidic cellular environment. Similar acidifications in the absence of a source of latent TGF-beta enhanced the positive growth response of 49F and AKR-2B cells to EGF and active TGF-beta and also the negative growth response of KiMSV-transformed 49F cells to active TGF-beta. These results are compatible with the idea that acidic cellular environments, particularly in tumor tissues, are conducive to activation of latent TGF-beta, perhaps in conjunction with other activating mechanisms, and to an enhanced response to some growth factors. However, the heterogeneity of cell populations within tumoral masses presents an obstacle to a clear understanding of the consequences of such activation.
MEDLINE INDEXING DATE: 198908
ISSN: 0020-7136
LANGUAGE: English
UNIQUE NLM IDENTIFIER: 89233486
CAS REGISTRY/EC NUMBER(S): 0 (Culture Media); 0 (Lactates); 50-21-5 (Lactic Acid); 62229-50-9 (Epidermal Growth Factor-Urogastrone); 76057-06-2 (Transforming Growth Factors); 9002-18-0 (Agar)

EXHIBIT C-13

List Contains 1 Item.
Current Search Formulation: +MERRILEES MJ; + 1990 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Endothelial cell stimulation of smooth muscle glycosaminoglycan synthesis can be accounted for by transforming growth factor beta activity.

ARTICLE SOURCE: Atherosclerosis (Netherlands), Apr 1990, 81(3) p255-65

AUTHOR(S): Merrilees MJ; Scott L

AUTHOR'S ADDRESS: Department of Anatomy, School of Medicine, University of Auckland, New Zealand.

MAJOR SUBJECT HEADING(S): Endothelium, Vascular [physiology]; Glycosaminoglycans [biosynthesis]; Muscle, Smooth, Vascular [metabolism]; Transforming Growth Factors [physiology]

MINOR SUBJECT HEADING(S): Cells, Cultured; Endothelium, Vascular [metabolism]; Sulfhydryl Compounds [pharmacology]; Swine; Transforming Growth Factors [metabolism]; Trypsin [pharmacology]

INDEXING CHECK TAG(S): Animal; Support, Non-U.S. Gov't

PUBLICATION TYPE: JOURNAL ARTICLE

ABSTRACT: Endothelial cell conditioned medium (ECCM) contains a factor which markedly stimulates smooth muscle cell (SMC) glycosaminoglycan (GAG) synthesis. We report here that the factor responsible is transforming growth factor beta (TGF-beta) as assessed by (1) protease and thiol sensitivity, (2) heat and acid enhancement of ECCM activity, and (3) neutralisation of ECCM activity by anti-TGF-beta-immunoglobulin. Anti-TGF-beta-neutralisation was effective against increases in both sulphated and non-sulphated GAG. Previous studies showed that ECCM from EC of varying densities stimulated individual GAG to varying degrees. ECCM from low density EC preferentially stimulated hyaluronic acid (HA) whereas ECCM from intermediate and high density cultures stimulated increasing amounts of sulphated GAG. Exposure of SMC to varying concentrations of TGF-beta produced a similar pattern Exposure of SMC to varying concentrations of TGF-beta produced a similar pattern of response. Very low amounts of TGF-beta (less than 10-500 pg/10 cells) stimulated a marked and significant increase in HA synthesis. Increase in chondroitin sulphate 4/6 was most marked at TGF-beta levels from 500-1000 pg/10(6) cells. At levels above 1000 pg/10(6) cells both HA and sulphated GAG synthesis decreased but still remained elevated above controls. These findings indicate that TGF-beta alone can account for the changes in SMC GAG synthesis stimulated by ECCM. It was also found, however, that heat-treated SMC conditioned medium stimulated SMC GAG synthesis, thus SMC may contribute to the control of their own GAG synthesis through autocrine TGF-beta activity.

MEDLINE INDEXING DATE: 199009

ISSN: 0021-9150

LANGUAGE: English

UNIQUE NLM IDENTIFIER: 90274739

CAS REGISTRY/EC NUMBER(S): EC 3.4.21.4 (Trypsin); 0 (Glycosaminoglycans); 0 (Sulfhydryl Compounds); 76057-06-2 (Transforming Growth Factors)

EXHIBIT C-14

List Contains 1 Item.
Current Search Formulation: +YOUNG SR; + 1990 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® (1999 Edition)

ARTICLE TITLE: Macrophage responsiveness to therapeutic ultrasound.
ARTICLE SOURCE: Ultrasound Med Biol (England), 1990, 16(8) p809-16
AUTHOR(S): Young SR; Dyson M
AUTHOR'S ADDRESS: Department of Anatomy, United Medical School, Guy's Hospital, London, England.
MAJOR SUBJECT HEADING(S): Macrophages [cytology]; Ultrasonic Therapy
MINOR SUBJECT HEADING(S): Cell Count; Cell Division; Cell Line; Cell Survival; Fibroblasts [metabolism]; Growth Substances [biosynthesis]; Macrophages [metabolism]
INDEXING CHECK TAG(S): Animal; Human
PUBLICATION TYPE: JOURNAL ARTICLE

ABSTRACT: Macrophages are a source of many important growth factors which can act as wound mediators during tissue repair. The aim of this work was to find out if levels of ultrasound which accelerate repair could stimulate the release of fibroblast mitogenic factors from an established macrophage-like cell line (U937). The U937 cells were exposed in vitro to continuous ultrasound at a space average, temporal average intensity of 0.5 W/cm2 at either 0.75 MHz or 3.0 MHz, for 5 min. The macrophage-conditioned medium was removed either 30 min or 12 h after exposure, and placed on 3T3 fibroblast cultures. Fibroblast proliferation (defined here as increase in cell number) was assessed over a 5-day period. The results showed that 0.75 MHz ultrasound appeared to be effective in liberating preformed fibroblast affecting substances from the U937 cells, possibly by producing permeability changes, whereas 3.0 MHz ultrasound appeared to stimulate the cell's ability to synthesize and secrete fibroblast mitogenic factors.
MEDLINE INDEXING DATE: 199109
ISSN: 0301-5629
LANGUAGE: English
UNIQUE NLM IDENTIFIER: 91247100
CAS REGISTRY/EC NUMBER(S): 0 (fibroblast-activating factor); 0 (Growth Substances)

EXHIBIT C-15

List Contains 1 Item.
Current Search Formulation: +SHULTZ PJ; + 1989 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® (1999 Edition)

ARTICLE TITLE: Mitogenic signals for thrombin in mesangial cells: regulation of phospholipase C and PDGF genes.
ARTICLE SOURCE: Am J Physiol (United States), Sep 1989, 257(3 Pt 2) pF366-74
AUTHOR(S): Shultz PJ; Knauss TC; Mene P; Abboud HE
AUTHOR'S ADDRESS: Department of Medicine, Veterans Administration Medical Center, Cleveland, Ohio.
MAJOR SUBJECT HEADING(S): Gene Expression Regulation; Glomerular Mesangium [physiology]; Mitogens [physiology]; Phospholipase C [genetics]; Platelet-Derived Growth Factor [genetics]; Thrombin [physiology]
MINOR SUBJECT HEADING(S): Calcium [metabolism]; Cytosol [metabolism]; Gene Expression Regulation [drug effects]; Glomerular Mesangium [cytology] [metabolism]; Mitogens [pharmacology]; Phosphatidylinositols [metabolism]; Proteins [metabolism]; RNA, Messenger [metabolism]; Thrombin [pharmacology]
INDEXING CHECK TAG(S): Human; Support, Non-U.S. Gov't; Support, U.S. Gov't, Non-P.H.S.; Support, U.S. Gov't, P.H.S.
PUBLICATION TYPE: JOURNAL ARTICLE

ABSTRACT: Thrombin is a proteolytic enzyme of diverse biological activities, which is produced during activation of the coagulation pathway. In addition, thrombin is a mitogen for fibroblasts and endothelial cells. Intraglomerular thrombosis and cell proliferation are common pathological features of several glomerular diseases. We studied the effect of thrombin on deoxyribonucleic acid (DNA) synthesis in cultured human mesangial cells and explored mechanisms of signal transduction involved. Bovine and human thrombin caused dose-dependent increases in DNA synthesis, inositol trisphosphate, and cytosolic calcium [(Ca2+)i]. A threefold increase in inositol-3-trisphosphate (IP3) levels was observed as early as 10 s after the addition of thrombin, whereas increases in (Ca2+)i occurred within 5-10 s and declined rapidly. Stimulation of mesangial cells by thrombin resulted in induction of messenger ribonucleic acids (mRNAs) encoding platelet-derived growth factor (PDGF) A- and B-chains. This was associated with an enhanced secretion of PDGF-like protein. These data provide mechanisms by which thrombin may regulate mesangial cell function in disease states.
MEDLINE INDEXING DATE: 198912
ISSN: 0002-9513
LANGUAGE: English
UNIQUE NLM IDENTIFIER: 89390640
CAS REGISTRY/EC NUMBER(S): EC 3.1.4.3 (Phospholipase C); EC 3.4.21.5 (Thrombin); 0 (Mitogens); 0 (Phosphatidylinositols); 0 (Platelet-Derived Growth Factor); 0 (RNA, Messenger); 7440-70-2 (Calcium)
GRANT ID NUMBER: DK-33665-DK-NIDDK; DK-07470-DK-NIDDK

EXHIBIT C-16

List Contains 1 Item.

Current Search Formulation: "electrical stimulation of growth "; + 1989 - All Articles; + 1990 - All Articles; + 1991 - All Articles; + 1992 - All Articles; + 1993 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Weak direct current accelerates split-thickness graft healing on tangentially excised second-degree burns.
ARTICLE SOURCE: J Burn Care Rehabil (United States), Jul-Aug 1991, 12(4) p285-93
AUTHOR(S): Chu CS; McManus AT; Okerberg CV; Mason AD Jr; Pruitt BA Jr
AUTHOR'S ADDRESS: Library Branch, United States Army Institute or Surgical Research, Fort Sam Houston, TX 78234-5012.
MAJOR SUBJECT HEADING(S): Burns [physiopathology]; Electric Stimulation Therapy; Skin Transplantation; Wound Healing [physiology]
MINOR SUBJECT HEADING(S): Burns [pathology] [surgery]; Cell Division; Guinea Pigs; Skin Transplantation [pathology]; Skin [pathology]; Transplantation, Autologous
INDEXING CHECK TAG(S): Animal; Male
PUBLICATION TYPE: JOURNAL ARTICLE

ABSTRACT: We have examined the effects of direct current (DC) conducted through silver-nylon dressings on the healing time and morphologic maturation of split-thickness grafts placed on tangentially excised deep partial-thickness burn wounds. Male guinea pigs (n = 120) were used as the experimental hosts. The DC-treated animals required 2 days for complete revascularization of their grafts; control animals required 7 days (p less than 0.01). The DC-treated animals had increased epithelial proliferation at the graft-wound interface as compared with controls (p less than 0.01). Grafts from DC-treated animals were firmly adherent within 4 days, whereas graft adherence in controls was weak before 7 days after grafting. At 3 months after grafting, control animal grafts had mild contraction with moderate hair loss and thick subepidermal fibrosis; the grafts in DC-treated animals expanded with the growth of the animals and had abundant hair growth and significantly reduced dermal fibrosis (p less than 0.01).
MEDLINE INDEXING DATE: 199202
ISSN: 0273-8481
LANGUAGE: English
UNIQUE NLM IDENTIFIER: 92042249

EXHIBIT C-17

EVIDENCE APPENDIX

ITEM NO. 18

Final Office Action issued May 5, 2008, page 14, paragraph 18

(also attached hereto as Exhibit B)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/064,000	04/21/1998	JAMES P. ELIA	796-P-12	5311

7590   05/05/2008

GERALD K. WHITE
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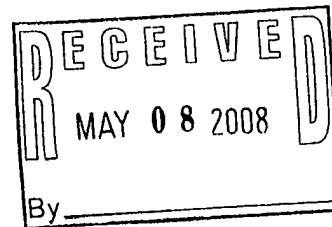| EXAMINER |
|---|
| GAMETT, DANIEL C |

ART UNIT	PAPER NUMBER
1647	

MAIL DATE	DELIVERY MODE
05/05/2008	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

RECEIVED
MAY 0 8 2008
By_____

	Application No.	Applicant(s)
	09/064,000	ELIA, JAMES P.
Office Action Summary	Examiner	Art Unit	
	DANIEL C. GAMETT	1647	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

> A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE <u>3</u> MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.
> - Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
> - If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
> - Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on <u>28 November 2007</u>.

2a)☒ This action is **FINAL**.   2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *403-405 and 407-412* is/are pending in the application.

    4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *403-405 and 407-412* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

    Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

    Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

    a)☐ All   b)☐ Some * c)☐ None of:

      1.☐ Certified copies of the priority documents have been received.

      2.☐ Certified copies of the priority documents have been received in Application No. _____.

      3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

    * See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1)☐ Notice of References Cited (PTO-892)

2)☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)

3)☐ Information Disclosure Statement(s) (PTO/SB/08)
    Paper No(s)/Mail Date _____.

4)☐ Interview Summary (PTO-413)
    Paper No(s)/Mail Date. _____.

5)☐ Notice of Informal Patent Application

6)☐ Other: _____.

DETAILED ACTION

1. The amendments of 11/28/2007 have been entered in full. Claims 1-402 and 406 are cancelled.

   The newly cancelled claims include claims which were rejected in previous office actions. All

   prior objection/rejections directed to cancelled claims are moot and hereby withdrawn.

2. Claims 403-405 and 407-412 are under examination.

Claim Rejections - 35 USC § 112

3. The following is a quotation of the second paragraph of 35 U.S.C. 112:

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   claim 403, step (b) "forming a bud" creates a lack of clarity as to whether the recited step

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   (p.7) that, "it is clear from the specification that the only step required by the practitioner is

   that of injecting stem cells into a selected site in a patient's body." Thus, Applicant

   acknowledges that although step (b) (and, by implication, step (c)) has the form of a method

   step, the actual intent is to recite an intended outcome. The claim defines the invention.

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the rejection in the '589 application have been deemed to be enabling for a scope that

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human patient comprising the steps of locally placing a CD34+ mononuclear cell harvested

from bone marrow or peripheral blood in a body of a human patient. Applicant suggests that

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class of growth factors that broadly and specifically includes genes, nucleic acids, a patient's

own cells (autologous cells), or universal cells, e.g., stem cells (global mononuclear bone

marrow cells), etc., all of which are described to promote tissue growth through

differentiation and morphogenesis." Applicant complains that the Examiner has only

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/064,000	04/21/1998	JAMES P. ELIA	796-P-12	5311

7590   05/05/2008

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EXAMINER
GAMETT, DANIEL C

ART UNIT	PAPER NUMBER
1647	

MAIL DATE	DELIVERY MODE
05/05/2008	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

RECEIVED
MAY 0 8 2008
By_____

	Application No.	Applicant(s)
Office Action Summary	09/064,000	ELIA, JAMES P.
	Examiner	Art Unit	
	DANIEL C. GAMETT	1647	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE <u>3</u> MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on <u>*28 November 2007*</u>.

2a)☒ This action is **FINAL**.   2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *403-405 and 407-412* is/are pending in the application.

    4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *403-405 and 407-412* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

    Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

    Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

    a)☐ All   b)☐ Some *   c)☐ None of:

        1.☐ Certified copies of the priority documents have been received.

        2.☐ Certified copies of the priority documents have been received in Application No. _____.

        3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

    * See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1)☐ Notice of References Cited (PTO-892)

2)☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)

3)☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.

4)☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

5)☐ Notice of Informal Patent Application

6)☐ Other: _____.

U.S. Patent and Trademark Office

PTOL-326 (Rev. 08-06)   Office Action Summary   Part of Paper No./Mail Date 20080227

DETAILED ACTION

1. The amendments of 11/28/2007 have been entered in full. Claims 1-402 and 406 are cancelled.

    The newly cancelled claims include claims which were rejected in previous office actions. All

    prior objection/rejections directed to cancelled claims are moot and hereby withdrawn.

2. Claims 403-405 and 407-412 are under examination.

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EVIDENCE APPENDIX

ITEM NO. 19

<u>The Journal of Invasive Cardiology</u>, Vol. 17, July 1, 2005,
entitled "Progenitor Cell Transplantation and Function
following Myocardial Infarction" (author unknown) cited by
the Examiner in the October 2, 2008 Office Action issued in
co-pending application Serial No. 09/836,750

(also attached hereto as Exhibit C—complete copy)

EXHIBIT D

COMPLETE COPY OF

July 1, 2005 publication in
<u>The Journal of Invasive Cardiology</u>, Vol. 17, entitled
"Progenitor Cell Transplantation and Function following
Myocardial Infarction" (author unknown)

cited by the Examiner in the November 20, 2008 Office Action

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Progenitor Cell Transplantation and Function following Myocardial Infarction

VOLUME: 17 PUBLICATION DATE: Jul 01 2005

Issue Number:
7

William O'Neill: I have a couple of questions for both of you. First, we are essentially talking about adult, autologous stem cells. As you all know, there is enormous controversy in the U.S. about the use of embryonic stem cells which we are currently prohibited from using. From a dispassionate, basic scientific approach, do you think there's an advantage to using embryonic stem cells, or will adult stem cells be sufficient in terms of their ability to replicate and cause myogenesis?

Peter Gonschior: After examining the data from experiences in other fields with vascular cells and their biological functions, I don't think there is a need for embryonic stem cells. The bone marrow cells appear to be very robust; even the non-randomized European trial data show that with a not-so-profound approach, it is possible to obtain positive effects. Due to the political scenery in Europe, I don't think it would be possible to conduct large clinical trials using embryonic stem cells.

Sigrid Nikol: I think we should try to optimize the strategies using autologous cells due to the ethical concerns. Due to these restrictions, there is not currently much proof available that embryonic stem cell therapy is that much better or even less risky. We know that these cells can differentiate, which means that they could differentiate into undesired cells as well. Thus, given the legal restrictions at present, we should stick with what we can do and try to make the best of it.

William O'Neill: Another key issue that arises from a clinical standpoint is that of direct injection versus intra-arterial or intravenous infusion. Do we know yet which would be the most efficient way — if the appropriate cell were to be found — to deliver the cells for the most efficient myogenesis?

Peter Gonschior: Sigrid and I have a lot of experience with local drug delivery. Efficiency of local drug delivery is in fact very low. It would be very smart to just inject the cells intravenously. But one critical issue with all of these data is that delivery efficiency, whether systemic or local, is very low. In fact, it turned out that endocardial delivery of these cells was the most efficient, resulting in the largest number of injected cells — up to about 30 million.

William O'Neill: The problem I have is that the stem cells are very sensitive to hypoxia, they need an oxygenated environment in order to thrive. Thus, if these cells are injected into a core infarct area, it will likely be hypoperfused and the ambient oxygen tension in that area may not be sufficient to support those cells. Sigrid, your experience involved a permanent ligation, right?

Sigrid Nikol: Actually, there were two sets of animals, but I only showed you those with the permanent ligation because there are currently more data on them. We also have a reperfusion model from 30 minutes after opening up the vessel.

William O'Neill: Did you find in the reperfusion model that there was any more myogenesis efficiency?

Sigrid Nikol: We perform stem cell therapy via bone marrow stimulation (not cell injection). At the moment, we do not have sufficient data on the reperfusion model. The problem is that when genes or cells are injected into the vessels, why should they pass the endothelium and migrate into the tissue where they are needed? It is very unlikely to happen, unless there is injured tissue. And with the venous application, there are the first-pass effects in the lungs and liver on top of it. These findings are not very convincing in my opinion, including the data from the Strauer group which lacked a truly randomized control group. The problem with intramyocardial injections into infarcted areas is a real one, as you stated, in that there is no blood/oxygen supply and potentially arrhythmogenic foci are created. Also, there may not be a homogeneous distribution of cells — an issue that has already been discussed with regard to gene therapy for the myocardium. Specifically, the question had been raised regarding how homogeneous gene therapy in the myocardium can be achieved without creating arrhythmias. With stem cells, this may be an even more important issue, as has been demonstrated in the work by Menasche et al.

William O'Neill: It seems to me that a highway is needed to get the cells to the tissue, and that's why we have been considering autoinfusion or direct intracoronary infusion of the agents, because in the reperfusion model, at least if there's an intact vascular structure, then the environment will be ambient and oxygenated until the cells can engraft. But my question is: Why would those cells stay at that target site? Also, we are lacking basic scientific understanding of the signals that allow the stem cells to hone in on that particular tissue. Since stem cells could potentially be coming in from the systemic circulation, we need to come up with a honing material that could be injected into the infarcted area and would allow the stem cells to accumulate there. We are moving forward in this field.

At the 2003 AHA meeting, data from the late-breaking BOOST trial were presented. It was, I think, a very well-conducted randomized trial involving 60 patients with placebo versus bone marrow infusion arms. The results showed a statistically significant increase in ejection fractions in the active treatment group compared with the placebo group. TOPCARE was a terrific study, but it was a sort of historical control study. Thus, we are beginning to accumulate data that suggest there is some merit to this approach. Let me turn this question over now to the other panelists. Matt Pollman, from Guidant Corporation, is on the panel. Matt, is there business potential in this field?

Matt Pollman: That's a great question, Bill. As a clinician and a scientist, this area is extremely

provocative and intriguing, particularly the data from the BOOST and TOPCARE myocardial infarction trials. There is an aggressive movement to push these trials out into more randomized, controlled, multi-center arenas. The issue for a company such as Guidant is what we can bring to the table and whether there is a business potential in this area. Confidentiality, of course, prevents me from saying too much here, but there certainly appear to be business opportunities for companies like ours. We believe that it will require a very safe delivery system that can be applied to a wide range of patients. The cells clearly need to be delivered at least very close to the target site in order to successfully do their job. In that light, an intracoronary infusion strategy raises questions about how to allow enough time for the cells to adhere, to transmigrate, enter the tissue and do their job. There is room for improvement in the delivery systems, and that's what Guidant is looking into. The TOPCARE acute myocardial infarction study headed up by Andreas Zeiher in Frankfurt uses a simple syringe injection system loaded with 10 cc of bone marrow, 3 cc of which is applied to the coronary arteries. The patients are then allowed to be in an unreperfused state for 3 minutes to allow the cells to do their job. Often, the limiting factor is that the patients complain of chest pain during the unreperfused state. Thus, there are opportunities to improve the current infusion protocol.

William O'Neill: We have been looking at it in a two-prong fashion, with a lot of interest in basic science on one end, and in clinical trials on the other end. As for the United States, if bone marrow is taken from a patient and then reinjected, the FDA considers it non-homologous use, even though it is not a drug or a commercial compound. The U.S. FDA regulations on stem cell use do not cover autologous use of these cells. Unfortunately, thus, this entire field has become embroiled in the embryonic stem cell and abortion debates. As a result, the FDA is incredibly cautious about allowing embryonic stem cell use. The FDA regulations state that if the stem cells are taken and not manipulated, and then reinjected, it falls outside of the FDA's authority. At the 2003 TCT meeting, the FDA representative specifically stated that if any compound is taken from the body and reinjected (i.e., spinal fluid) into an area where it doesn't normally need to be, then the FDA does have regulatory authority.

Also, the FDA insists on receiving a significant amount of data — and rightfully so — on basic safety issues such as clotability and other matters surrounding the infusion of these cells into the coronary arteries. I think it will be another year or two in the U.S. before the basic science data are available to allow clinical trials to proceed. The bulk of the basic scientific studies will be carried out in South America and Europe.

Having said that, we have a perfect opportunity right here to learn from our colleagues about where this field is headed internationally. I know that you, Alfredo, are very much in the midst of all of this research. Would you mind telling us what your group will be doing in terms of your randomized trial?

Alfredo Rodriguez: Thank you, Bill. We are just starting a randomized trial that will follow the rules of the TOPCARE MI trial. Our trial involves 40 patients, 20 in each arm. One patient arm receives autologous bone marrow injections. These are acute myocardial infarction patients from 3 to 12 hours after symptom onset; all patients receive percutaneous coronary intervention and stenting. After reperfusion, we randomize the patients on day-4. On day-5, we puncture the patient's iliac crest, and the next day, we infuse the drug in 10 ml of solution into the coronary arteries.

The patient undergoes angiography immediately following the PCA procedure. Global and regional ejection fractions are measured. An acute and 4-month dobutamine stress echocardiogram is then done, followed by an MRI and SPECT imaging.

Our institution has a very active bone marrow transplant team. The hematologists who serve on our trial's executive committee told us that it was not necessary to place this trial under the Argentinian equivalent of the FDA, because autologous bone marrow is not a drug; it's not foreign material to the body. Thus, our trial is approved by the local transplant agency. My concern involves legal problems that could arise. I would like to hear from Sigrid and the other European colleagues here if the ongoing clinical trials in Europe in this field are approved by their respective regulatory agencies, or if they are only approved as protocols by the local hospitals' scientific committees, with the patients of course providing informed, written consent.

Sigrid Nikol: According to the blood transfusion and federal drug laws, there are certain regulatory approvals needed, particularly if the doctor obtaining the cells is not the same doctor using them therapeutically. In this case, cells are considered a blood product and their use is regulated.

Alfredo Rodriguez: I do know that the TOPCARE trial did not have local German regulatory approval.

Richard Heuser: I assume that we're talking here about a normal 10 cc bone marrow aspirant — no filtering — just administering it down the coronary arteries. Is that correct? And then the balloon is inflated for 3 minutes or so to allow the cells to disperse? And several injections are given?

Alfredo Rodriguez: Yes, 3 or 4 injections are given.

William O'Neill: This approach has generated controversy due to the fact that the bone marrow is unfiltered and thus contains fat, spicules, mesenchymal cells, and so on. Basically, the injection contains the "kitchen sink" and we hope that the right cells go to the right place and do the right thing. The other argument is that we know which cells we want, so we should just take them, filter them, grow them in media and replicate them, increase their efficiency, and then inject them. Those are the two schools of thought on the subject, but I can't tell you which is the correct one, because we might not have the right cells. It may be that the CD34 positive cells are not the right ones. In the TOPCARE study, they actually took both the peripheral cells and the cells obtained through leukophoresis, then identified them, segregated them, and grew them in a culture medium to increase their numbers.

In terms of FDA regulations, whenever you manipulate and produce cells, a commercial product results, and thus clearly falls under the FDA's purview. A regulatory "gray" area still exists in the U.S. when it comes to simply taking cells, leukophoresing them, removing the stem cells, and reinfusing them.

Richard Heuser: The first time I saw this technique presented by the group in Frankfurt, I was astonished at how simple it actually was. I am surprised that I didn't get into regulatory trouble myself about 5 or 6 years ago when I treated a patient in the middle of the night who tore a coronary artery. At that time, I had our home-made covered stents and some JoMed stents, but the vessel was 2.3 mm, and the patient was in cardiogenic shock. I had administered ReoPro and t-PA to this 70-year-old female patient. I just took some clot, combined it with a little of the protamine, and it got to be enough of a slurry. I then put it down with a balloon, occluded the vessel, then re-opened it — and it was sealed. I then stented the vessel, and it was fine. But I find it hard to believe that if we administer these bone marrow cells to a patient with a huge infarct that we could get into trouble with the FDA. Some of these therapies make good sense for the individual patient, but more study data are needed.

William O'Neill: Let me pose a question to Paul Overlie, who has had extensive experience treating acute myocardial infarction patients for the past 20 years. Is there a need for this, Paul? The CADILLAC and recent myocardial infarction studies showed a 2% mortality rate and an ejection fraction mean of about 50%. How often does the situation arise that would warrant going to the trouble of doing bone marrow aspirates and leukophoresis on these patients?

Paul Overlie: The very high-risk, no-reflow patients might benefit from these therapies. Once these bone

marrow cells are aspirated, is there some way to get the cytokines activated before injecting them, or concentrating them, so that the U.S. FDA would approve of the technique?

William O'Neill: Now that more studies have been conducted since the last time — about a year-and-a-half ago — that the FDA was approached about this, it's likely that the FDA will show more interest in allowing U.S. clinical trials to go forward. Phil, do you think there's an application for the genetic or stem cell repair approaches in the general vasculature — the aorta or peripheral vasculature?

Philip Walker: I am a peripheral vascular surgeon, so I am definitely interested in myocardial repair to get our patients fit for intervention or following infarcts after intervention. There are a number of emerging areas where the approach might be helpful. Peter mentioned the single-center study on stem cell use for peripheral revascularization which involves an area where patients are nonreconstructible, particularly diabetics with renal failure.

I also wonder whether the no-reflow phenomenon — perhaps even in the setting of acute limb ischemia — might benefit from stem cell therapy. Stem cells may also be useful as an adjunct to tissue engineering. I work with a group who are developing a biological graft based on a peritoneal growth, which may be another useful area for the adjunctive use of stem cells. This therapy is being developed with the aim of improving the antithrombotic effects, which might also apply to prosthetic grafts that have been plagued by thrombotic problems when small diameters are involved.

Aortic repair in patients who have not yet developed sizable aneurysmal disease may be yet another area for stem cell therapy, but we need to learn how to identify these patients. We also need to learn to identify patients with small aneurysms, as stem cell repair might be useful in repairing and inhibiting the process in these patients.

Another viable area may be in the area of stroke and revascularization, as well as brain repair. This raises the issue of whether the mechanisms will be generic across all of those vascular beds, or whether differences exist, and whether the basic science needs to be worked out for the different areas.

I would like to ask about the issue of toxicity, particularly in diabetic patients in whom there may be an acceleration of diabetic retinopathy, tumorigenesis in the elderly patients, as well as plaque instability. Are these issues relevant?

William O'Neill: Perhaps because these bone marrow cells are pluripotential and have stimuli for differentiation, they will probably not be carcinogenic. And since they serve repair processes, it is unlikely that they will cause pathologic proliferation. Those are all critical questions that have plagued the field of gene therapy in which vectors were found that caused some cells to proliferate wildly. I think these cells will be safer, but we really won't know until a large number of patients are treated.

From our own acute myocardial infarction work, I presented a slide on the number of patients who present within 2 hours of symptom onset, and that number is about 5% of the U.S. acute myocardial infarction population — at least with the current standards. Perhaps with more novel, patient-directed approaches, this percentage could rise. After 2 hours, whether the patient is reperfused or not, there will be a substantial amount of necrotic tissue and a large permanent infarct zone. If stem cell therapy could be safely applied, I believe that many patients could benefit in terms of improved regional function, making an akinetic anterior wall hypokinetic, or improving or preventing aneurysm formation.

Brian O'Murchu: Has the coronary sinus retrograde perfusion route been used for administration of these cells?

William O'Neill: Not that I am aware. There is one company that makes a device for access to the coronary sinus, and then needle injection into the myocardium. I think there may be some interest in using that as an access site rather than performing ventricular puncture.

Brian O'Murchu: I was just talking with my colleague, Alex Zapolanski, about whether the solution emerges from the ostia of the arterial coronaries during retrograde coronary perfusion, and of course it does. Thus, it would seem to provide the opportunity to 'bathe' the myocardium through the use of a system that can be balloon-occluded, allowing perfusion to be maintained.

William O'Neill: That has been discussed, but I am not aware of any ongoing trials on that topic.

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EVIDENCE APPENDIX

ITEM NO. 20

<u>The Journal of Invasive Cardiology</u>, Vol. 17, July 1, 2005, entitled "Progenitor Cell Transplantation and Function following Myocardial Infarction" (author unknown) cited by the Examiner in the October 2, 2008 Office Action issued in co-pending application Serial No. 09/836,750

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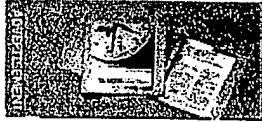
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Invasive Cardiology News Wire

Progenitor Cell Transplantation and Function following Myocardial Infarction

VOLUME: 17 PUBLICATION DATE: Jul 01 2005

Issue Number:
7

William O'Neill: I have a couple of questions for both of you. First, we are essentially talking about adult, autologous stem cells. As you all know, there is enormous controversy in the U.S. about the use of embryonic stem cells which we are currently prohibited from using. From a dispassionate, basic scientific approach, do you think there's an advantage to using embryonic stem cells, or will adult stem cells be sufficient in terms of their ability to replicate and cause myogenesis?

Peter Gonschior: After examining the data from experiences in other fields with vascular cells and their biological functions, I don't think there is a need for embryonic stem cells. The bone marrow cells appear to be very robust; even the non-randomized European trial data show that with a not-so-profound approach, it is possible to obtain positive effects. Due to the political scenery in Europe, I don't think it would be possible to conduct large clinical trials using embryonic stem cells.

Sigrid Nikol: I think we should first try to optimize the strategies using autologous cells due to the ethical concerns. Due to these restrictions, there is not currently much proof available that embryonic stem cell therapy is that much better or even less risky. We know that these cells can differentiate, which means that they could differentiate into undesired cells as well. Thus, given the legal restrictions at present, we should stick with what we can do and try to make the best of it.

William O'Neill: Another key issue that arises from a clinical standpoint is that of direct injection versus intra-arterial or intravenous infusion. Do we know yet which would be the most efficient way — if the appropriate cell were to be found — to deliver the cells for the most efficient myogenesis?

Peter Gonschior: Sigrid and I have a lot of experience with local drug delivery. Efficiency of local drug delivery is in fact very low. It would be very smart to just inject the cells intravenously. But one critical issue with all of these data is that delivery efficiency, whether systemic or local, is very low. In fact, it turned out that endocardial delivery of these cells was the most efficient, resulting in the largest number of injected cells — up to about 30 million.

William O'Neill: The problem I have is that the stem cells are very sensitive to hypoxia; they need an oxygenated environment in order to thrive. Thus, if these cells are injected into a core infarct area, it will likely be hypoperfused and the ambient oxygen tension in that area may not be sufficient to support those cells. Sigrid, your experience involved a permanent ligation, right?

Sigrid Nikol: Actually, there were two sets of animals, but I only showed you those with the permanent ligation because there are currently more data on them. We also have a reperfusion model from 30 minutes after opening up the vessel.

William O'Neill: Did you find in the reperfusion model that there was any more myogenesis efficiency?

Sigrid Nikol: We perform stem cell therapy via bone marrow stimulation (not cell injection). At the moment, we do not have sufficient data on the reperfusion model. The problem is that when genes or cells are injected into the vessels, why should they pass the endothelium and migrate into the tissue where they are needed? It is very unlikely to happen, unless there is injured tissue. And with the venous application, there are the first-pass effects in the lungs and liver on top of it. These findings are not very convincing in my opinion, including the data from the Strauer group which lacked a truly randomized control group. The problem with intramyocardial injections into infarcted areas is a real one, as you stated, in that there is no blood/oxygen supply and potentially arrhythmogenic foci are created. Also, there may not be a homogeneous distribution of cells — an issue that has already been discussed with regard to gene therapy for the myocardium. Specifically, the question had been raised regarding how homogeneous gene therapy in the myocardium can be achieved without creating arrhythmias. With stem cells, this may be an even more important issue, as has been demonstrated in the work by Menasche et al.

William O'Neill: It seems to me that a highway is needed to get the cells to the tissue, and that's why we have been considering autoinfusion or direct intracoronary infusion of the agents, because in the reperfusion model, at least if there's an intact vascular structure, then the environment will be ambient and oxygenated until the cells can engraft. But my question is: Why would those cells stay at that target site? Also, we are lacking basic scientific understanding of the signals that allow the stem cells to hone in on that particular tissue. Since stem cells could potentially be coming in from the systemic circulation, we need to come up with a honing material that could be injected into the infarcted area and would allow the stem cells to accumulate there. We are moving forward in this field.

At the 2003 AHA meeting, data from the late-breaking BOOST trial were presented. It was, I think, a very well-conducted randomized trial involving 60 patients with placebo versus bone marrow infusion arms. The results showed a statistically significant increase in ejection fractions in the active treatment group compared with the placebo group. TOPCARE was a terrific study, but it was a sort of historical control study. Thus, we are beginning to accumulate data that suggest there is some merit to this approach. Let me turn this question over now to the other panelists. Matt Pollman, from Guidant Corporation, is on the panel. Matt, is there business potential in this field?

Matt Pollman: That's a great question, Bill. As a clinician and a scientist, this area is extremely provocative and intriguing, particularly the data from the BOOST and TOPCARE myocardial infarction trials. There is an aggressive movement to push these trials out into more randomized, controlled, multi-center arenas. The issue for a company such as Guidant is what we can bring to the table and whether there is business potential in this area. Confidentiality, of course, prevents me from saying too much here, but there certainly appear to be business opportunities for companies like ours. We believe that it will require a very safe delivery system that can be applied to a wide range of patients. The cells clearly need to be delivered at least very close to the target site in order to successfully do their job. In that light, an intracoronary infusion strategy raises questions about how to allow enough time for the cells to adhere, to transmigrate, enter the tissue and do their job. There is room for improvement in the delivery systems, and that's what Guidant is looking into. The TOPCARE acute myocardial infarction study headed up by Andreas Zeiher in Frankfurt uses a simple syringe injection system loaded with 10 cc of bone marrow, 3 cc of which is applied to the coronary arteries. The patients are then allowed to be in an unreperfused state for 3 minutes to allow the cells to do their job. Often, the limiting factor is that the patients complain of chest pain during the unreperfused state. Thus, there are opportunities to improve the current infusion protocol.

William O'Neill: We have been looking at it in a two-prong fashion, with a lot of interest in basic science on one end, and in clinical trials on the other end. As for the United States, if bone marrow is taken from a patient and then reinjected, the FDA considers it non-homologous use, even though it is not a drug or a commercial compound. The U.S. FDA regulations on stem cell use do not cover autologous-use of these cells. Unfortunately, thus, this entire field has become embroiled in the embryonic stem cell and abortion debates. As a result, the FDA is incredibly cautious about allowing embryonic stem cell use. The FDA regulations state that if the stem cells are taken and not manipulated, and then reinjected, it falls outside of the FDA's authority. At the 2003 TCT meeting, the FDA representative specifically stated that if any

Stemi Interventions ▸

EVIDENCE APPENDIX

ITEM NO. 21

The Journal of Invasive Cardiology, Vol. 17, July 1, 2005,
entitled, "Tissue Engineering and Interventional Cardiology"
cited by the Examiner in the October 2, 2008 Office Action
issued in co-pending application Serial No. 09/836,750

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Tissue Engineering and Interventional Cardiology

VOLUME: 17 PUBLICATION DATE: Jul 01 2005

Issue Number:

7

author:

Speaker: David Holmes, MD
Moderator: Reginald Low, MD
Panel members: George Dangas, MD, Wolfgang Ritter, MD, Peter Gonschior, MD, Brian Firth (Cordis Corporation)

George Dangas: There have been many attempts and many failures in this field due perhaps to the inherent tendency of interventional cardiologists to move quickly and work from assumptions, applying what may not yet be well understood. Gene therapy and engineered viruses (associated viruses, attenuated viruses, etc.) are examples of this. When it became clear that we were unable to identify the most appropriate and effective agent for angiogenesis, we looked toward the newly fashionable stem cell-based therapies. Even researchers make 3 million agents, and 2 of them turn out to be effective, that would be fine. On the other hand, perhaps the stem cells will produce 2 or 3 agents that work for angiogenesis, but at the same time, 1 or 2 other agents produce negative effects — the result being that the positive effect hoped for is not achieved.

Thus, the interventional cardiology field must achieve more "crisp" results based on more "crisp" basic science, with better-established findings, in order to better understand what the targets are and pursue them in a more methodological manner. Our methodology needs to be evidence-based, as opposed to the focusing on the practicalities of how to achieve our aims. We need to scale down the in vivo applications and return to the laboratory.

David Holmes: There are a number of small, randomized trials currently under way, primarily in Europe. Perhaps some of our European colleagues here could discuss these trials. We are already in the middle of human trials before obtaining adequate scientific data about which specific cells to use, how many cells, when to deliver them, and how to deliver them. Is that a good thing? What if they fail? does that mean the approach is wrong? Or does it mean that we were doing it incorrectly?

Peter Gonschior: The good thing is that very robust cells are used based on solid, basic scientific data. That led to the application of a large variety of cells, which led to what appeared to be good data. The patient data, such as ejection fraction, however, are not terribly impressive. Ejection fraction improvement is not very significant, especially when you factor in the amount of energy wasted to achieve any clinical impact in the patients. More basic, relevant data are required to guide us toward the best approach.

Wolfgang Ritter: We have never used drug-eluting stents, so we wait to see what the cardiologist does.

David Holmes: From the industry standpoint, it would be nice to have a patentable product so that the product's unique design bearing the corporate name could be marketed. Given that God invented the progenitor cells and has a pretty strong patent on them, how do we "patent" a cell? For example, a bone marrow cell injected in the coronary artery — how do you design a device to do that? How do you make a living at that, since most any device could do that task?

Brian Firth: Let me come at this from a different angle. For some time now, Cordis has looked at what it already had as facilitating technology. We have been on the delivery side of the business, specifically with our Noga systems, the NogaStar®, the MyoStar™ injectable catheter, and so on. Thus, the mapping, definition and ability to deliver something in a very site-specific manner constitute the piece of the business Cordis has focused on. Having said that, in order to obtain 510-K FDA device approval, we must prove that it actually does something. Thus, Cordis is currently working on the area of autologous bone marrow with stem cells. Our interest is not in trying to figure out how to patent stem cells, which can't be done, but rather in the delivery of these cells, because we think that a more local delivery system would be better than a more general one. Cordis seeks to design a system, thus, that would deliver the cells that have been identified for their contractile properties to a site that has been defined as compromised.

Richard Heuser: These are expensive studies to conduct, thus, if the product is not patentable, it will not attract industry funding. In the case of the Bioheart study, how will this trial be conducted? Will stem cells be given, or no cells, or a small number of them? Also, we want to target the patient population that is not eligible for heart transplants. We have been talking about bone marrow cells as well. My understanding is that there is a very good possibility that these cells can be delivered intravenously with the same results. So how do we design, say, a skeletal muscle cell study that would actually end up garnering FDA approval for the therapy? And what about bone marrow — is it really necessary to go down the coronary arteries and go selectively into the myocardium?

David Holmes: Those are two important questions. Bioheart is a skeletal muscle myoblast product. The company considered this product a drug when it applied to the FDA for approval. In drug trials, the FDA requires data on ineffective dosage in addition to a toxic dose, and a couple of doses that do work. Thus, the first dose in the Bioheart project that was approved by the FDA was absolutely ineffective — it might as well have been placed under the patient's pillow. The data that came out of Europe on Bioheart involved a much higher dose, albeit with a small number of patients.

In terms of the second point as it relates to where and how to administer the cells, some information has

shown that when these different sorts of cells are delivered intravenously, they go to the lungs and have a "tremendous time," and they don't reach the myocardium. So while it makes perfect sense to use the intravenous approach, these cells are filtered out in the lungs and remain there. If those cells are active and produce cytokines, perhaps that's all we would want to use them for. Maybe these cells aren't the magic solution, and maybe we don't have a clue about this. Perhaps we can use these cells for the cytokines they produce systemically and they will cause other bone marrow cells to hone in on the site of injury. But at the present, we just don't know enough about this process.

Patrick Whitlow: I just want to give you an update on Bioheart because of their underlying disease process, these patients are very prone to arrhythmia and sudden death. And theoretically, if you are adding islands of tissue in the left ventricle that is already damaged, these islands of tissue are not enervated in the same way as the surrounding tissue and the conduction properties aren't the same. You would theorize that this could set up re-entry circuits. Thus, ventricular arrhythmia presents an enormous problem in terms of conducting studies because many of these patients are going to die from their underlying disease. To detect if cell injection causes worsened arrhythmia will be very difficult, but a potentially serious problem. Therefore, the first v.s. clinical trial involves patients who already have defibrillators, and the number of patients will be small because of the need for defibrillators. The study should answer the question of whether this is arrhythmogenic — which Patrick Serruys believes is the case. Other researchers in France don't believe that injecting cells is arrhythmogenic. Who knows? It will take a long time and a lot of patients to arrive at the answer.

If a start-up company tries to make this therapy work, it will be very difficult for industry to actually fund the research from start to finish. We know from the animal studies that efficacy increases with higher doses of cell therapy, but we have yet to find what a potentially toxic dose is for the size of the island of cells that produce arrhythmias. I think that the bulk of this research will have to be federally funded. It's all very interesting work, and according to the animal models, it should work in humans.

David Holmes: Although the skeletal myoblasts appear to be arrhythmogenic, it appears to relate to engraftment properties. With true stem cells — whatever they are — it doesn't seem to be as problematic, whether because there have so far been very small numbers of patients, or whether indeed these stem cells are more pluripotential and engraft better, or whether they are more homogeneously distributed, and aren't just islands. It is early in this field, and I would echo what George said: in a field where so much rides on a product or technique, some of the trials are too premature because we often don't have the necessary solid scientific underpinnings before launching an important large trial. The biggest potential problem downstream to this approach is that if the product fails, we don't know for certain whether failure was due to the product's ineffectiveness or because we didn't know how to properly use it.

George Dangas: I would like to comment on interpreting the data from some of these early studies. I don't think we have the proper tools to accurately study the early results. The preliminary decision by the Rotterdam group was to implant defibrillators in all patients of the Bioheart study after two or three deaths occurred in one arm. Still, we haven't figured out whether it was actually the number of implants or if it was a patient substrate with a number of implants that caused the arrhythmogenecity. I think that any other study at this stage would produce statistical errors in both directions, which makes it very difficult to determine whether it was a failure of the agent, the liver system, or that the patients in the treatment arm were too sick and were going to die anyhow. That last explanation is a possibility because, due to ethical considerations, we usually enroll "no option" patients for these types of agents.

Richard Heuser: The Bioheart study involves a specific, patentable therapy which provides greater incentive to the company to see the project through to the end. One thing that always concerns me is determining what the endpoint will be. We all love to see those ejection fractions, but I think that the two main endpoints will likely be treadmill time (endurance) and objective findings of symptom relief. A third endpoint might be the number of hospitalizations for congestive heart failure. I agree that we have to conduct this study in some sort of randomized fashion. I think that the low-dose cells which we discussed will be a good way to do it. Also, since it's a very small number of patients being subjected to this very expensive therapy, I wonder if we could collect data on the patients before we commence therapy. In other words, we would assign the patient. We all know how long it takes to enroll patients in this trial; there's a lot of information to gather. During the six-month lead-in period, more data points could be obtained by looking at retrospective data on those individual patients. It won't be enough to see the ejection fraction increase, and there certainly won't be a reduction in mortality.

David Holmes: I think there will be a reduction in mortality rates and it will be the lead-in phase. For instance, all of the transplant centers have patient deaths while on the waiting list. This study will provide the same type of information. There may be other endpoints — viability of MR, for example. Whether viability with MR will be an "approvable" endpoint remains to be seen, however. We will need to be creative in terms of endpoints.
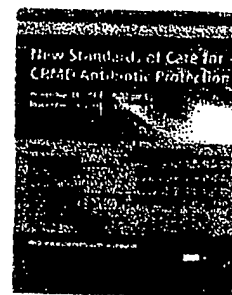
William O'Neill: I agree with you, George, in terms of the degree of our ignorance about the basic science in this area. My own feeling is that God — or nature — in His infinite wisdom, is a lot smarter that we will be for a few centuries yet in terms of the cascade of processes that actually allow a new cell to come in and regenerate. It is a little foolhardy to say that we should wait until we completely understand these processes before any clinical trials are launched. These early attempts are fine, as long as patients aren't harmed and as long as the patients are properly selected in terms of their ability to spontaneously improve function. As you said, pre-transplant patients will not improve function and there will likely be a big upside and very little downside for them. I would thus encourage conducting these small, mechanistic trials as a means of enlightening us as to where we stand and where we must go. Finally, when we change from the basic experiments to human trials, we are dealing with patients who are on all types of medications. Do ACE inhibitors, calcium channel blockers, beta-blockers and nitrates alter, improve or decrease the ability of cells to regenerate? We simply don't know the answer to this question. I do believe that we face a long process of trial and error, and will make small advances along the way.

David Holmes: I think that view is correct, provided that if the small trials are negative, we don't then abandon the field and decide that the therapy doesn't work. It seemed to be the case with some of the gene therapy trials where incredible hype was followed by randomized trials that produced negative results, setting the field back significantly. I think that well-designed studies aimed at identifying mechanisms will be terribly important for the field.

Brian Firth: In terms of endpoints, I believe that this falls under the same rules as most of the heart failure studies. The FDA wants to see that therapies designed for patients with heart failure or impending heart failure don't increase mortality while improving other parameters. Thus, researchers don't have to prove that the therapy improves survival rates, but they do have to prove that it doesn't adversely affect survival. That was the big lesson learned from the inotropic therapy studies.

Thomas McNamara: What has been the progress and/or expectations with other critical organs — namely, the liver and the kidneys? Has work been done in this area?

David Holmes: I think work has been done, particularly on the liver, partly because it can regenerate. We tend to think that heart cells will repair what has been a problem, and I don't know if they will wildly proliferate and make a totally new heart, liver cells can do. You need to understand that I'm not exactly

sure where in the abdomen the liver resides, so I'm perhaps not the best person to ask!

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EVIDENCE APPENDIX

ITEM NO. 22

The Journal of Invasive Cardiology, Vol. 17, July 1, 2005,
entitled, "Tissue Engineering and Interventional Cardiology"
cited by the Examiner in the October 2, 2008 Office Action
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Tissue Engineering and Interventional Cardiology

VOLUME: 17 PUBLICATION DATE: Jul 01 2005

Issue Number:
7

author:

Speaker: David Holmes, MD
Moderator: Reginald Low, MD
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EVIDENCE APPENDIX

ITEM NO. 23

Murry et al. 1996 publication in <u>J. Clin. Invest.</u> entitled, "Skeletal Myoblast Transplantation for Repair of Myocardial Necrosis" cited by the Examiner in the November 28, 2003 Office Action issued in co-pending application Serial No. 09/836,750

(also attached hereto as Exhibit E)

Skeletal Myoblast Transplantation for Repair of Myocardial Necrosis

Charles E. Murry,* Robert W. Wiseman,‡ Stephen M. Schwartz,* and Stephen D. Hauschka§

*Department of Pathology, ‡Department of Radiology, and §Department of Biochemistry, University of Washington School of Medicine, Seattle, Washington 98195

Abstract

Myocardial infarcts heal by scarring because myocardium cannot regenerate. To determine if skeletal myoblasts could establish new contractile tissue, hearts of adult inbred rats were injured by freeze-thaw, and $3–4.5 \times 10^6$ neonatal skeletal muscle cells were transplanted immediately thereafter. At 1 d the graft cells were proliferating and did not express myosin heavy chain (MHC). By 3 d, multinucleated myotubes were present which expressed both embryonic and fast fiber MHCs. At 2 wk, electron microscopy demonstrated possible satellite stem cells. By 7 wk the grafts began expressing β-MHC, a hallmark of the slow fiber phenotype; coexpression of embryonic, fast, and β-MHC continued through 3 mo. Transplanting myoblasts 1 wk after injury yielded comparable results, except that grafts expressed β-MHC sooner (by 2 wk). Grafts never expressed cardiac-specific MHC-α. Wounds containing 2-wk-old myoblast grafts contracted when stimulated ex vivo, and high frequency stimulation induced tetanus. Furthermore, the grafts could perform a cardiac-like duty cycle, alternating tetanus and relaxation, for at least 6 min. Thus, skeletal myoblasts can establish new muscle tissue when grafted into injured hearts, and this muscle can contract when stimulated electrically. Because the grafts convert to fatigue-resistant, slow twitch fibers, this new muscle may be suited to a cardiac work load. (J. Clin. Invest. 1996. 98:2512–2523.) Key words: myocardial infarction • skeletal myoblast • myosin heavy chain • contractile function • cell transplantation

Introduction

Experimental and clinical therapies for myocardial infarction have focused traditionally on limiting infarct size. Unfortunately, the goal of limiting myocardial injury has been difficult to achieve clinically, because ischemic myocardium dies quite rapidly (1) and most patients wait more than 3 h after coronary occlusion before seeking medical attention. As an alternative approach, we are exploring strategies to induce the injured heart to heal with muscle replacement rather than forming scar tissue.

One strategy for muscle regeneration is to transplant either skeletal or cardiac myocytes into the injured heart. Studies from Field's group showed that cardiac myocytes can be transplanted into normal hearts, where they couple with host cardiocytes via intercalated discs (2, 3). However, a major drawback to using cardiocytes is their inability to proliferate in culture. At present it seems unlikely that enough primary cardiocytes could be obtained from the patient or histocompatible donor to repair a myocardial infarct in humans. On the other hand, skeletal muscle satellite cells (muscle stem cells) proliferate well in culture. Satellite cells could be obtained from muscles of infarct patients and rapidly expanded in culture, or stocks of potentially therapeutic myoblasts could be obtained from embryos and frozen for subsequent use (4, 5). Furthermore, physiological studies have shown that when properly conditioned, skeletal muscle can adapt to perform a cardiac-type work load (6). Recent studies have demonstrated the feasibility of grafting skeletal myoblast lines into normal hearts (7) and autologous satellite cells into injured hearts (8, 9). However, to generate significant amounts of functional new muscle the transplanted cells ideally should proliferate and then differentiate into mature myofibers capable of sustaining a cardiac work load. This study was performed to determine the proliferation and differentiation patterns of skeletal myoblasts after engraftment into injured rat hearts and to determine whether this new muscle could support contractile activity.

Methods

Skeletal myoblast isolation and culture. These studies were approved by the University of Washington Animal Care Committee and were conducted in accordance with federal guidelines. Skeletal myoblasts were obtained from the limbs of 1–3-d-old Fischer rats. This inbred strain was used to avoid immune barriers to transplantation. After time of killing, the carcasses were skinned and the limbs were placed into cold tissue culture media. Under a dissecting microscope, the muscles were stripped of surrounding adipose tissue and fascia and bluntly dissected from their tendons. The muscles were minced with iridectomy scissors until a fine slurry was formed. The slurry was then digested in 0.05% trypsin/EDTA (GIBCO-BRL, Gaithersburg, MD) in Ham's saline A at 37°C, with intermittent mechanical agitation to assist dispersal. After 30–45 min the cell suspension was filtered through sterile gauze to remove undispersed tissue fragments and rod shaped mature myofibers. Cells were plated at ~5 × 10⁶ cells/dish in 100-mm gelatinized plates in 10 ml Ham's F10C media, containing 15% horse serum and 50 μg/ml gentamicin sulfate (Gemini Bioproducts, Inc., Calabasas, CA). Recombinant human basic fibroblast growth factor was added twice daily to a final concentration of 6 ng/ml, and the complete medium was replaced once per day. Approximately 10% of the cells attached and grew with a doubling time of ~18 h. The cultures contained a mix of small, oval myoblasts and elongated, spindle-shaped cells consistent with fibroblasts. Subconfluent cultures were passaged every 2–3 d (1:5 split) to minimize the occurrence of myogenic differentiation at higher density. On the day before transplantation, the cultures were tagged for subsequent identification in vivo. In some experiments cells were tagged with fluorescent micro-

spheres (1:500 dilution of stock 200 nm yellow-green fluorescent microspheres; Molecular Probes, Eugene, OR). The latex microspheres were endocytosed (typically > 20 spheres/cell) and served as cytoplasmic markers (10). In other experiments, cells were incubated overnight with [^{3}H]thymidine (1 μCi/ml) to mark their nuclei after autoradiography. Cultures were trypsinized immediately before transplantation and suspended at a concentration of $\sim 3 \times 10^7$/ml. Small aliquots of the remaining cell suspension were replated at $\sim 2 \times 10^4$ cells/cm^2 into gelatinized, multichamber plastic slides, and fixed in methanol after various culture intervals for immunostaining.

Rat cardiac injury models. Inbred male Fischer rats (Simonsen Labs, Gilroy, CA) weighing 350–400 grams were anesthetized with intraperitoneal ketamine-xylazine (68 and 4.4 mg/kg, respectively), intubated, and mechanically ventilated with room air. The heart was exposed aseptically via a left thoracotomy, and a 1-cm-diameter aluminum rod, precooled with liquid nitrogen, was placed in direct contact with the anterior left ventricle for 15 s. Freeze-thaw reproducibly caused a disc-shaped region of coagulation necrosis, ~ 1 cm in diameter, extending ~ 2 mm into the myocardium. It should be noted that while infarcts typically have irregular borders with viable peninsulas of subepicardial myocardium along penetrating vessels, freeze-thaw lesions consist of confluent necrosis in the subepicardium with viable myocardium in the subendocardium. Because they are not transmural, freeze-thaw lesions do not cause cardiac aneurysm formation. Despite these differences, the cellular patterns of coagulation necrosis, inflammation and phagocytosis, granulation tissue formation, and scarring after freeze-thaw injury are indistinguishable from myocardial infarction (11–13), making it a suitable model to study myocardial repair.

In the initial studies, $\sim 3 \times 10^6$ myoblasts in 100 μl tissue culture media were injected superficially into the center of the injured region immediately after injury, using a 27-gauge needle. Then, the chest was closed and the rats were allowed to recover for timed intervals from 1 d to 3 mo (n = 4/time point). To mimic a clinical situation more closely, a second protocol was used in which the freeze-thaw lesion was allowed to heal for 1 wk before transplanting myoblasts. By 1 wk, most of the necrotic myocardium had been replaced by granulation tissue, but scar formation had not yet begun. The rats (n = 2/time point; no 3 d or 3 mo time points) were reanesthetized and a thoracotomy was repeated. The heart was exposed and a 100-μl suspension containing $\sim 3 \times 10^6$ myoblasts was injected into the wound as described above. The chest was closed and the animals were allowed to recover for intervals from 1 d to 7 wk.

To detect DNA synthesis in the grafts the rats were treated with the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU)[1] (Boehringer-Mannheim, Indianapolis, IN). 1 d before time of killing, the rats were lightly anesthetized, and a 50-mg tablet of BrdU was implanted subcutaneously for measurement of cell replication. Preliminary studies showed that a subcutaneous 50-mg BrdU tablet gave comparable replication rates to a 24-h continuous infusion with an osmotic mini-pump (not shown). For rats killed 1 d after transplantation, a single 10-mg pulse of BrdU was given intraperitoneally 1 h before time of killing. This avoided incorporation of BrdU into the cells which were cycling at the time of transplantation.

Rats were killed with a pentobarbital overdose and their hearts were excised. In the immediate transplantation groups, the aorta was cannulated and the hearts were perfusion fixed with methyl Carnoy's solution (60% methanol, 30% chloroform, 10% glacial acetic acid), transversely sectioned, and embedded in paraffin by routine methods. In groups transplanted 1 wk after injury, the hearts were transversely sectioned, embedded in OCT (Miles Inc., Kankakee, IL), and frozen in a dry ice–ethanol bath for frozen section analysis. In both protocols, sections of gut were obtained as controls for measurement of cell replication with BrdU.

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1. *Abbreviations used in this paper:* BrdU, 5-bromo-2'deoxyuridine; MHC, myosin heavy chain.

Measurement of contractile function in isolated wound strips. Rat hearts were given 4.5×10^6 myoblasts (n = 8) in 100 μl or a sham injection of saline (n = 3) immediately after injury. 2 wk after engrafting, the hearts were excised and transversely sectioned. Under a dissecting microscope, most of the subendocardial myocardium was trimmed away from the injured region, and isolated wound strips ($\sim 1.5 \times 1.5 \times 8$ mm) were prepared. One or two strips were studied from each myoblast-engrafted heart, and two or three strips were studied from each sham-injected heart. The strips were ligated at both ends with silk suture and then placed in a bath of physiological saline with the following composition (mmol/liter): 116 NaCl, 4.6 KCl, 1.2 MgSO$_4$, 2.5 CaCl$_2$, 26 Mops (pH 7.4), 11 glucose, and 10 mg/liter gentamicin. The buffer was equilibrated with 95% O$_2$/5% CO$_2$ and maintained at 20°C via a thermostatically controlled water jacket. Wound strips were mounted between an isometric force transducer (model 60-2995; Harvard Apparatus, Inc., South Natick, MA) and a fixed glass hook. Resting tension was set initially at 0.5 g. Strips were stimulated with 1-ms bipolar pulses delivered via platinum wire electrodes using a Grass model S48 stimulator (Astro-Med, Inc., West Warwick, RI). Voltage was increased in 10-V increments until contractile activity was observed. Force traces were displayed on a digital storage oscilloscope (model 3091; Nicolet Instrument Corp., Madison, WI) and recorded using a General Scanning model RS4-5P strip chart recorder. After determining the force–voltage relationship, the optimal length for force production was determined for each wound strip using test contractions at 2-min intervals, a time sufficient for metabolic recovery in mammalian fast twitch muscles (14). Force–frequency analysis was performed by increasing the stimulation frequency in 1-Hz increments; tetanus was defined as the point where the oscillations of contractile force at the plateau were < 3% of the net force generated (14). Finally, to test fatigability the grafts were subjected to a simulated cardiac-like duty cycle, consisting of 0.33 s of tetanus followed by 0.67 s of relaxation (1:2 cycle), continuing for 6 min. After completion of functional studies the strip's cross-sectional area was determined, and the tissue then was processed for histology or electron microscopy.

Immunocytochemistry. Antibodies used for immunostaining are given in Table I. 6-μm frozen sections were cut on a cryostat, briefly air dried, and stored at −70°C until use. 5-μm paraffin sections were deparaffinized in xylene and rehydrated in a graded alcohol series. Cultured cells were fixed and stored in cold PBS until use. For all samples, endogenous peroxidase activity was quenched by incubating with 0.3% H$_2$O$_2$ in methanol for 30 min. Immunostaining was carried out at room temperature. Sections were blocked with 1.5% normal horse serum in PBS for 1 h. The sections were then incubated with the primary antibody in 1.5% horse serum for 1 h, followed by incubation with the secondary antibody (rat adsorbed horse anti–mouse, 1:400 dilution; Vector Labs. Inc., Burlingame, CA) for 1 h. Antigens were localized with an avidin-biotin-peroxidase complex (ABC Elite kit; Vector Labs). For staining with a single antibody, diaminobenzidine (Sigma Immunochemicals, St. Louis, MO) was used as a chromadine. For double immunolabeling with antibodies to myogenic substrate. For double immunolabeling with antibodies to myogenic substrate. For double immunolabeling with antibodies to myogenic substrate sin and BrdU, sections were first exposed to 1.5 N HCl for 15 min at 37°C to denature the DNA, followed by a rinse in 0.1 mol/liter borax to stabilize the denatured strands. Sections were then stained routinely for myosin heavy chain (MHC) using diaminobenzidine. After a second quenching in 0.3% H$_2$O$_2$, sections were blocked with 1.5% normal horse serum, and then incubated with a mouse monoclonal antibody to BrdU for 1 h. After incubation with the secondary antibody (horse anti–mouse), BrdU was localized with an avidin-biotin-peroxidase complex, using True Blue (KPL, Gaithersburg, MD) as substrate. Cross-reactivity between the first primary antibody and the second secondary antibody did not occur, as long as the True Blue substrate was incubated for a short duration (< 1 min). Sections were counterstained either with methyl green, nuclear fast red, or hematoxylin.

Electron microscopy. After measurement of contractile function, one of the tissue strips was immersed in half strength Karnovsky's fix-

Antibody	Antigen recognized	Dilution	Source	Reference
MF-20	Sarcomeric MHCs	Hyb. Sup., 1:100	American Type Culture Collection, Rockville, MD	39
MY-32	Skeletal MHC-fast (types IIA and IIB)	Mouse ascites, 1:2000	Sigma Immunochemicals	40
BA-G5	Cardiac MHC-α	Hyb. Sup., 1:5	American Type Culture Collection	41
F1.652	Embryonic MHC	Hyb. Sup., 1:100	Developmental Studies Hybridoma Bank*	42
A4.951	β-MHC	Hyb. Sup., 1:50	American Type Culture Collection	43
Anti-BrdU	BrdU	IgG, 1:50000	Eurodiagnostics, Apeldoorn, The Netherlands	44

IgG, purified IgG monoclonal antibody; *Hyb. Sup.*, hybridoma supernatant. *The monoclonal antibody F1.652, developed in the laboratory of Dr. Helen Blau, was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, and the Department of Biological Sciences, University of Iowa, Iowa City, IA, under contract NO1-HD-2-3144 from the National Institute of Child Health and Human Development.

ative and dissected into small cubes < 1 mm in greatest dimension. The tissue was fixed overnight in half strength Karnovsky's fixative, postfixed for 1 h in 1% osmium tetroxide at room temperature, dehydrated through a graded alcohol series followed by propylene oxide, and embedded in Medcast resin (Ted Pella, Inc., Redding, CA). Semithin sections were stained with toluidine blue and examined by light microscopy. Thin sections were cut from selected blocks, stained with lead citrate and uranyl acetate, and examined in a Jeol JEM 1200EXII transmission electron microscope. Representative areas were photographed.

Results

Characteristics of myoblast cultures. The muscle cultures contained a mixed cell population. At least 22% of the cells were skeletal muscle, as indicated by their staining for sarcomeric myosin after switching to a differentiation medium containing 1.5% serum and no FGF for 3 d. This procedure underestimates the true percentage of skeletal muscle cells by severalfold, since the nonmyogenic cells continue to divide after the medium switch while the myoblasts complete their present cell cycle and then terminally differentiate. Approximately 1% of the cells stained with antibodies to smooth muscle α-actin, which can mark either smooth muscle cells or fibroblasts. Virtually none of the cells stained with an antibody for the endothelial marker von Willebrand factor. The remaining cells were presumably fibroblasts.

Histology and differentiation patterns of myoblast grafts. Cultured skeletal myoblasts were transplanted into cardiac freeze-thaw lesions either immediately after injury, or, to mimic a clinical situation more closely, cells were transplanted 1 wk after injury. The two protocols yielded similar results and will be described together; minor differences are noted below. On the first day after transplantation the myoblasts were mononuclear cells (Fig. 1 *A*). The grafted cells could be distinguished clearly from inflammatory cells within the necrotic tissue by their larger size and characteristic oval shape. (Fibroblast ingrowth from the surrounding tissue had not yet begun at this time.) The identity of the grafted cells was confirmed by their cytoplasmic fluorescent microspheres and radioactive nuclei (not shown). Mitotic figures were common. The grafted cells did not stain with antibodies to skeletal or cardiac MHCs (Fig. 1 *B*). Thus, muscle differentiation had not yet occurred.

By 3 d after transplantation, many of the grafted cells had fused to form multinucleated myotubes (Fig. 1 *C*). Myotubes were partially aligned along the short (transverse) axis of the

heart. The myotubes stained with antibodies to sarcomeric MHC, embryonic MHC (Fig. 1 *D*), and to MHC-fast (not shown). Occasional cross-striations were noted, but these were not frequent at this time (Fig. 1 *D*). The myotubes did not express cardiac MHC-α. By 1 wk the grafts were easily recognizable as skeletal myofibers and many cells contained cross-striations. As before, the new myofibers stained with antibodies to sarcomeric MHC, embryonic MHC, and MHC-fast, but did not express cardiac MHC-α (not shown). By 2 wk after transplantation the grafts had the appearance of maturing skeletal myofibers (Fig. 1 *E*). Sarcomeres were well formed, and many cells had peripheral nuclei. The myofibers stained intensely with antibodies to sarcomeric myosin, embryonic MHC (Fig. 1 *F*), and skeletal MHC-fast (Fig. 1 *G*). No staining with cardiac MHC-α antibodies was observed at 2 wk.

At 7 wk after transplantation the grafts were islands of mature skeletal muscle within young scar tissue (Fig. 1, *H–J*). There was a moderate increase in cell diameter compared with 2 wk. None of the muscle grafts were infiltrated or splayed apart by scar tissue, nor was there evidence of fiber atrophy. Vascular density appeared normal for muscle tissue (Fig. 1 *J*). All of the 7-wk grafts stained strongly with antibodies to sarcomeric myosin and embryonic MHC (Fig. 1 *H*). The grafts injected immediately after injury stained intensely with antiskeletal MHC-fast, comparable with Fig. 1 *F*. In contrast, the grafts injected 1 wk after injury stained poorly with antiskeletal MHC-fast (see below). No staining with antibodies to cardiac MHC-α was observed in the grafts, while the adjacent myocardium stained intensely (Fig. 1 *I*).

At 3 mo after transplantation the grafts again had the appearance of mature skeletal muscle (Fig. 1 *K*). Most myofibers had peripheral nuclei, and vascular density appeared normal. Fiber diameter was generally larger than in the 7-wk group, indicating that the cells had hypertrophied between 7 wk and 3 mo (compare Fig. 1, *J* and *K*). In one heart, however, part of the graft was infiltrated by scar tissue which encircled individual myofibers and was associated with fiber atrophy (Fig. 1 *L*). The grafts continued to express embryonic MHC and MHC-fast (not shown). Once again, no staining with antibodies to cardiac MHC-α was observed (comparable with Fig. 1 *I*). At all time points the myofibers were predominantly aligned parallel with the short (transverse) axis of the heart and therefore appeared in longitudinal section. However, some fascicles of muscle appeared obliquely or cross-sectioned in this plane.
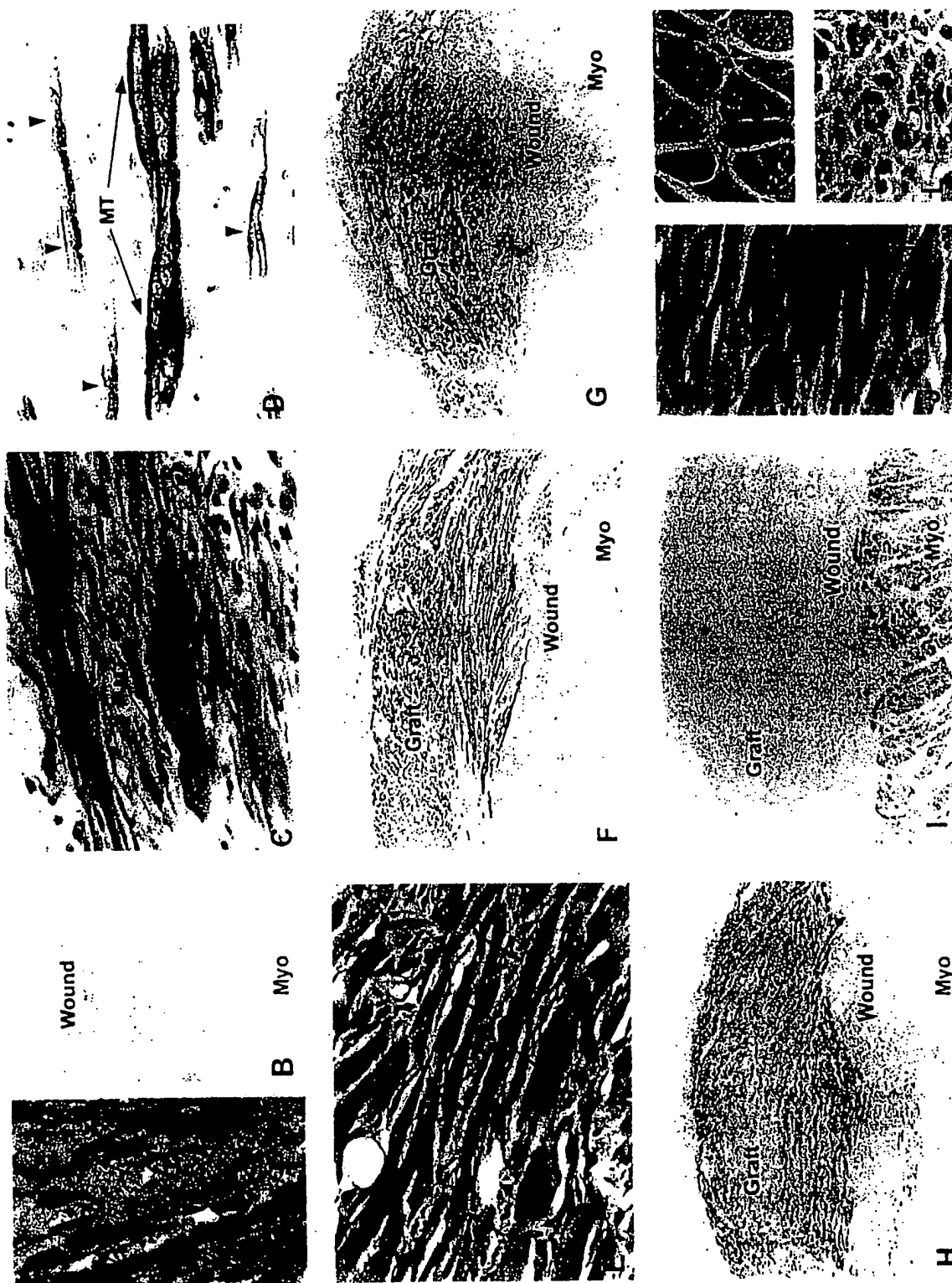
In summary, the grafts began to differentiate into myo-

Figure 1. Morphology and MHC expression in skeletal myoblast grafts. Rat hearts were injured by freeze-thaw and syngeneic skeletal muscle cells were grafted into the lesions. All panels in this figure are from hearts which were grafted immediately after injury. (*A*) 1-d graft. The grafted cells are identifiable as relatively large, oval shaped cells (*arrows*) within the necrotic myocardium. One graft cell is in mitosis (*arrowhead*). Numerous smaller inflammatory cells are present within the lesion. Fibroblast ingrowth from surrounding viable tissue had not yet begun at this

tubes between 1 and 3 d and acquired the appearance of maturing myofibers with well formed sarcomeres by 2 wk. The grafts expressed both embryonic MHC and MHC-fast at all times between 3 d and 3 mo. There was no expression of cardiac MHC-α at any time.

Electron microscopy. Electron microscopy was performed on one heart, 2 wk after myoblast grafting. Most of the grafted cells had well formed, slightly contracted sarcomeres which were aligned in registry (Fig. 2 *A*). Mitochondria were abundant. Multinucleation was evident in many cells, as were well formed T-tubules. However, in other cells a spectrum of morphological stages was present, consistent with developing skeletal muscle (Fig. 2 *B*). Cells at the earliest stage were small, had scanty myofibril content, and contained focal aggregations of electron-dense material suggestive of developing Z-discs. In these cells there were abundant ribosomes and glycogen, a prominent Golgi apparatus, and dilated segments of sarcoplasmic reticulum. Intermediate cells were larger and had increasing amounts of myofibrils with a corresponding decrease in ribosomes and glycogen. Some cells had well formed sarcomeres, but these were out of registry compared with the most mature cells. No intercalated discs were identified between cells in the graft region. Adjacent myofibers often had intimately apposed, interdigitating cell membranes. Occasional cells were identified with electron-dense membrane structures suggestive of intermediate adherens junctions and gap junctions (Fig. 2, *C* and *D*). Some mature myofibers were closely associated with mesenchymal cells, located within the basal lamina compartment of the myofiber. Their location within the basal lamina of the myofiber suggests that they might be new satellite stem cells (Fig. 2, *E* and *F*). Some of these mesenchymal cells had abundant rough endoplasmic reticulum, similar to fibroblasts. Cells with this morphology have also been described in regenerating skeletal muscle by Trupin et al. (15). Their location within the basal lamina of the myofiber and the

absence of collagen in this space make it unlikely that these cells are actually fibroblasts.

Myoblast grafts convert from fast to slow twitch fibers. The poor staining for MHC-fast in the 7-wk group with delayed transplantation seemed at variance with the morphology of the grafts, which showed relatively hypertrophic cells with well formed sarcomeres. We hypothesized that the grafts had undergone fiber type conversion to slow twitch muscles, which no longer expressed high levels of MHC-fast. Slow twitch fibers have physiological similarities to cardiac muscle, including a high capacity for oxidative phosphorylation and fatigue resistance. Furthermore, slow fibers use β-MHC as a major contractile protein, which is also the predominant myosin in developing rat hearts. In contrast, fast twitch fibers use glycolysis for ATP production, have a low aerobic capacity and fatigue rapidly, and do not express β-MHC (16). Therefore, we compared β-MHC expression with skeletal MHC-fast, to determine fiber types in the maturing grafts.

At 1 wk the grafts stained intensely for MHC-fast (Fig. 3 *A*) but did not stain with an antibody to β-MHC (Fig. 3 *B*). At 2 wk the grafts continued to express MHC-fast. In the group transplanted immediately after injury no expression of β-MHC was noted at 2 wk, yet in grafts transplanted 1 wk after injury some cells expressed β-MHC (not shown). At 7 wk after transplantation the two groups differed in expression of MHC-fast, with strong staining in the immediate transplant group (see Fig. 1 *G*) and weak staining in the group where transplantation was delayed for 1 wk after injury (Fig. 3 *C*). However, both the immediate and delayed transplantation groups exhibited extensive staining for β-MHC at 7 wk after transplantation (Fig. 3 *D*). At 3 mo there was continued expression of β-MHC and MHC-fast in the immediate transplantation group; we did not study the delayed transplantation protocol at 3 mo. Thus, myoblast grafts appeared to be undergoing conversion from fast twitch to slow twitch fibers. Conversion appeared to take place

time. Hematoxylin and eosin stain. ×800. (*B*) Low magnification of 1-d graft stained for embryonic MHC. The freeze-thaw lesion (*Wound*) occupies approximately the upper 75% of the field, while residual subendocardial myocardium (*Myo*) is present in the lower 25%. None of the grafted cells express embryonic MHC, indicating no differentiation had taken place yet. Methyl green counterstain. ×80. (*C*) 3-d graft. Multiple multinucleated myotubes (*MT*) are present. Note that myotubes are already aligned in parallel. The surrounding tissue contains numerous fibroblasts (some of which may be of graft origin), macrophages, and capillaries, characteristic of granulation tissue. Two mitotic figures are present at the lower right (*arrowheads*). Hematoxylin and eosin stain. ×800. (*D*) 3-d graft stained for embryonic MHC. The multinucleated myotubes (*MT*) express embryonic MHC, indicated by brown staining. Note faint cross-striations present at the periphery of some myotubes (*arrowheads*). Comparable staining was seen using antibodies to MHC-fast (not shown). Methyl green counterstain. ×800. (*E*) 2-wk graft. Multinucleated myofibers are present and many have peripherally placed nuclei (*arrows*); most of these nuclei appear to be within the sarcolemma, although some may be immediately external. Cross-striations were readily seen under the microscope but appear faint in the photograph. Hematoxylin and eosin staining. ×800. (*F*) 2-wk graft stained for embryonic MHC. The myofibers of the graft stain vigorously for embryonic MHC, while the underlying granulation tissue (*Wound*) and subendocardial myocardium (*Myo*) do not stain. Methyl green counterstain. ×80. (*G*) 2-wk graft stained for fast fiber isoforms of MHC. There is intense staining of the engrafted myofibers (*Graft*), indicating that they exhibit a fast twitch phenotype. Note that the residual myocardium (*Myo*) beneath the graft does not stain, nor does the granulation tissue of the injured region (*Wound*). ×80. (*H*) 7-wk graft stained for embryonic MHC. The graft continues to stain vigorously for embryonic MHC. There is no staining in the underlying young scar tissue (*Wound*) or the residual subendocardial myocardium (*Myo*). Methyl green counterstain. ×80. (*I*) 7-wk graft stained for cardiac MHC-α. The skeletal myofibers of the graft do not express MHC-α, nor does the underlying scar tissue (*Wound*). This indicates that the grafted skeletal muscle does not show cardiac differentiation. The subendocardial myocardium (*Myo*) stains vigorously for MHC-α. Methyl green counterstain. ×80. (*J*) 7-wk graft. Mature myofibers are present. Most myofibers have peripheral nuclei. Cross-striations were readily apparent under the microscope, but again are faint in the photograph. Multiple capillaries are present within the muscle tissue (*arrows*). Hematoxylin and eosin stain. ×800. (*K*) 3-mo graft. The myofibers (obliquely and cross-sectioned) have peripheral nuclei and are closely apposed with little intervening extracellular matrix. The myofibers are hypertrophic compared with the 7-wk grafts (compare fiber diameter with *J*). Most 3-mo grafts had this appearance. Hematoxylin and eosin stain. ×800. (*L*) 3-mo graft. The myofibers (cross-sectioned) in this region are encased by dense scar tissue and are atrophic. Note the markedly diminished cell diameters compared with *K*. Such entrapment of myofibers by scar was seen in one region of one heart. Hematoxylin and eosin stain. ×800.
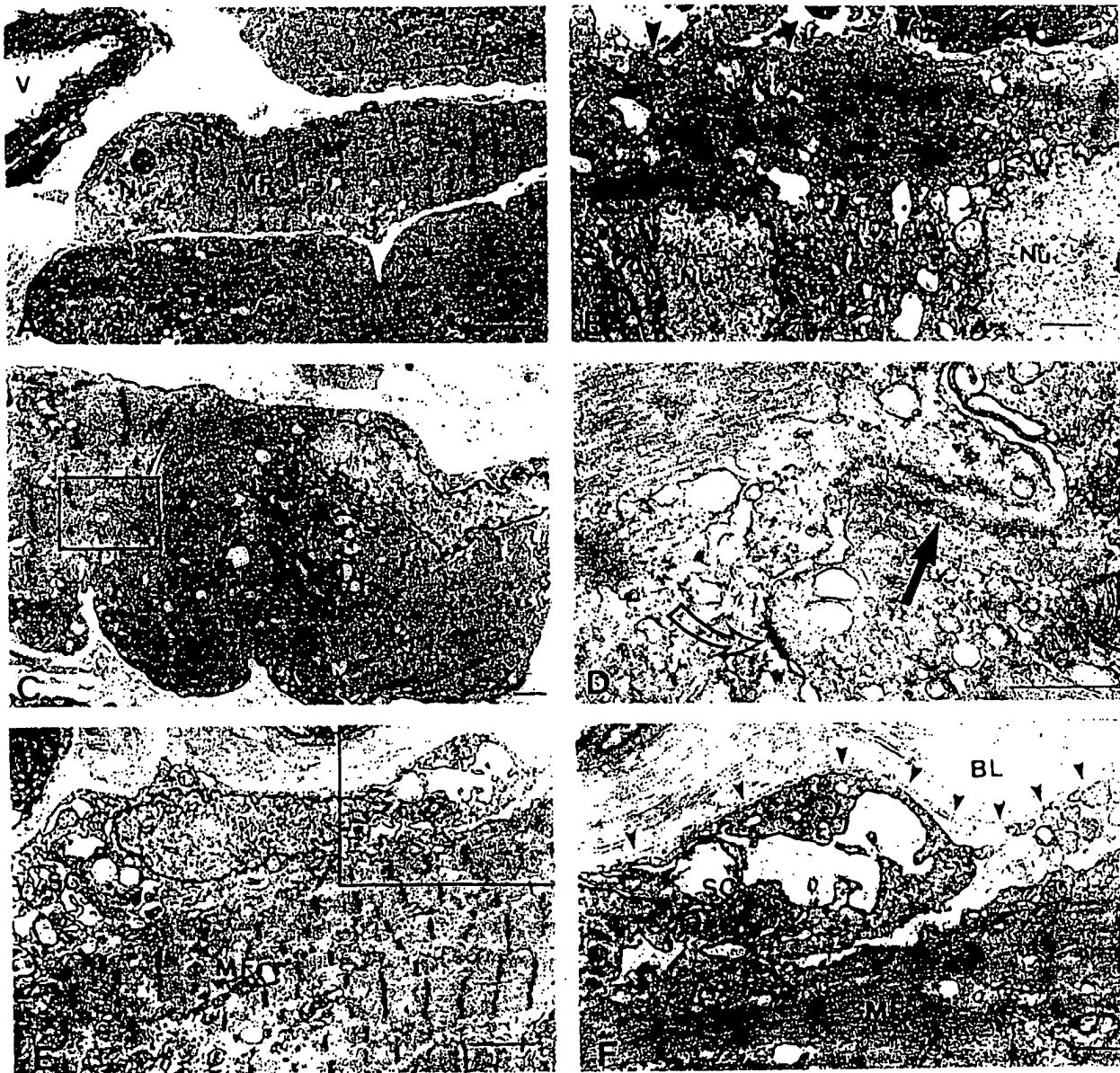
Figure 2. Transmission electron micrographs of 2-wk-old myoblast graft. The graft was placed immediately after cardiac freeze-thaw injury. (*A*) Low magnification overview showing well differentiated, striated skeletal myofibers (*MF*) within a collagen-rich matrix. A small venule (*V*) is shown at the left aspect. *Nu*, nucleus. Bar, 5 μm. (*B*) Moderately differentiated skeletal myofiber containing two nuclei (*Nu*), a modest complement of myofibrils (*mf*), and abundant ribosomes and sarcoplasmic reticulum between the nuclei. The sarcolemma is delimited by arrowheads. Bar, 1 μm. (*C*) Intercellular junction formation between adjacent myofibers. The two cells have closely apposed and interdigitated membranes. Two electron-dense plaques between the cells are present within the boxed region, suggestive of an adherens type intermediate junction and a gap junction, shown at higher magnification in *D*. Bar, 1 μm. (*D*) Higher magnification of the junctional region boxed in *C*, showing putative intermediate junction between adjacent myofibers (*solid arrow*) and gap junction (*open arrow*). Bar, 0.5 μm. (*E*) Skeletal myofiber (*MF*) with closely apposed mesenchymal cell atop it, suggestive of a satellite cell (*SC*). The boxed region is shown at higher magnification in *F*. Bar, 2 μm. (*F*) Higher magnification of region boxed in *E*. The putative satellite cell (*SC*) and the myofiber (*MF*) are contained within the same basal lamina compartment (*BL*, outlined by *arrowheads*). Although the cell has abundant rough endoplasmic reticulum, its location within the basal lamina of the myofiber and the absence of fibrillar collagen from this space make it unlikely that this is a fibroblast. Bar, 1 μm.

more rapidly when cells were transplanted into an injury with more advanced healing.

Proliferation of myoblast grafts. To identify cells undergoing DNA synthesis, the thymidine analogue BrdU was administered for 24 h before time of killing in most groups; animals in the day 1 group received a single pulse of BrdU 1 h before time of killing. Double immunostaining was performed with antibodies to the fast isoform of MHC and to BrdU, to detect
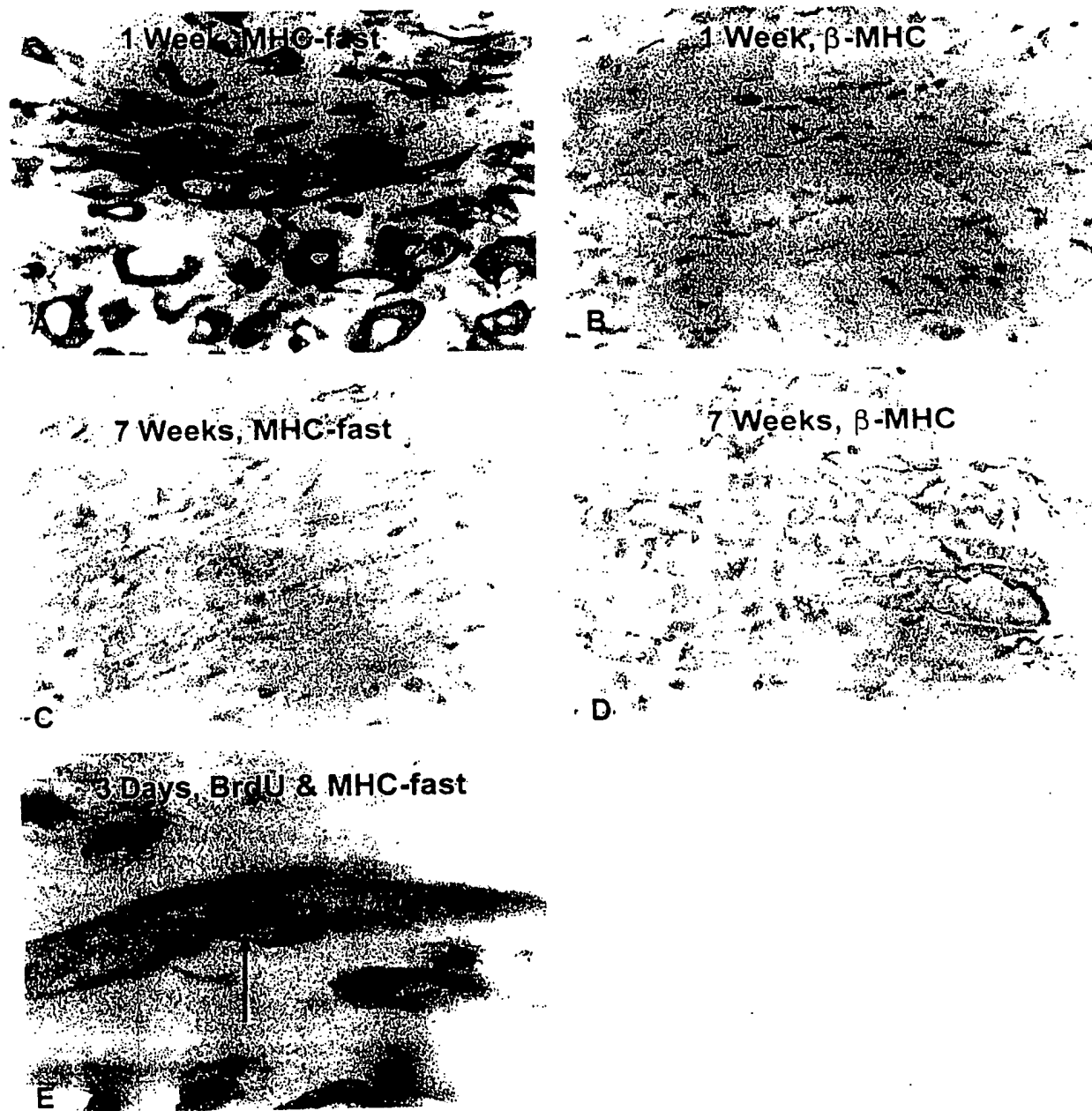
Figure 3. Fiber type conversion and proliferation of engrafted skeletal myoblasts. For the fiber typing experiments, rat hearts were injured by freeze-thaw and the lesions were allowed to heal for 1 wk. Syngeneic skeletal myoblasts were engrafted into the 1-wk-old wounds. For studies of cell proliferation, myoblasts were engrafted immediately after cardiac injury. Rats were killed at the indicated times after transplantation. Antibodies specific to fast twitch (MHC-fast) and slow twitch (β-MHC) fibers were used to define fiber types. Processing for frozen sections in A–D resulted in formation of contraction bands, artifactual clumping of the sarcomeres due to hypercontracture. BrdU was administered 24 h before time of killing to detect DNA synthesis. Double immunostaining for BrdU and MHC was then performed on paraffin sections. Appearance of a BrdU-positive nucleus within a myosin-positive cell indicated the myoblast had replicated and fused into the myotube within the last 24 h. (A) 1-wk graft stained for fast fiber isoforms of MHC. There is intense staining of the engrafted cells, indicating a fast fiber phenotype. Hematoxylin counterstain. ×960. (B) 1-wk graft stained with an antibody to the slow fiber-specific β-MHC. None of the grafted cells express β-MHC at this time, indicating that the cells show no characteristics of slow fibers. Methyl green counterstain. ×960. (C) 7-wk graft stained with an antibody to MHC-fast. There is weak staining compared with the 1-wk graft (A). Methyl green counterstain. ×960. (D) 7-wk graft stained with an antibody to β-MHC. The grafted cells now express β-MHC, indicating that they are acquiring a slow fiber phenotype (compare with B). Methyl green counterstain. ×960. (E) 3-d graft doubly stained for BrdU (purple) and MHC-fast (brown). One nucleus within the myotube stains purple (arrow), indicating it has undergone DNA replication before fusion into the myotube. The remaining nuclei in the myotube do not contain BrdU and pick up the red counterstain. Numerous myosin-negative cells in the surrounding wound tissue also stain positively for BrdU. Nuclear fast red counterstain. ×2,400.

A Twitch   4.0 Hz   8.0 Hz   Control

Time (In sec)

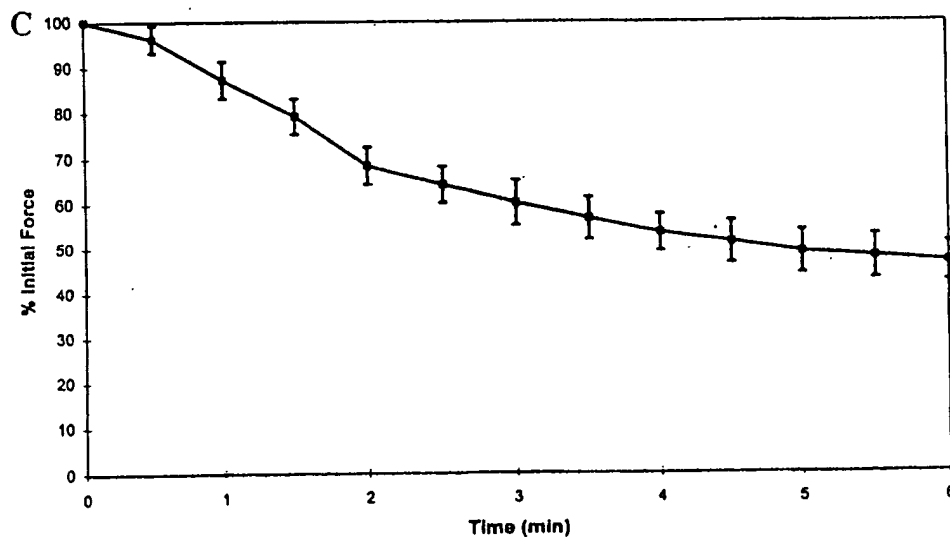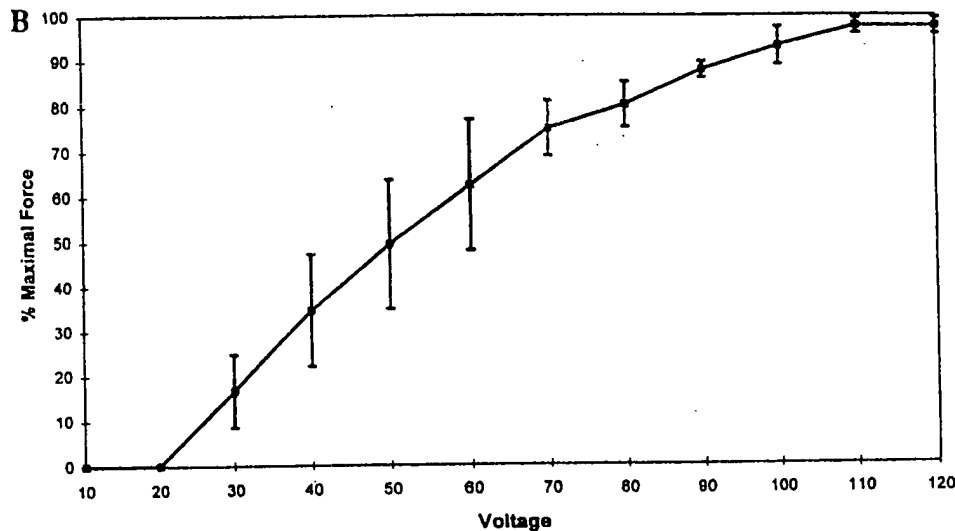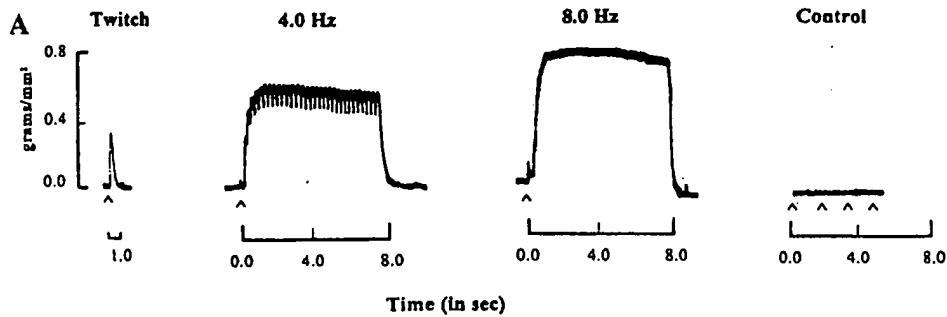B   % Maximal Force   Voltage

C   % Initial Force   Time (min)

Figure 4. Contractile function of myoblast grafts ex vivo. Wound strips from injured hearts receiving either skeletal myoblasts or a sham saline injection were excised at 2 wk. Wounds were mounted on an isometric tension myograph in oxygenated buffer and electrically stimulated. The carats indicate the onset of electrical stimulation. Force has been normalized to cross-sectional area. (*A*) The first panel shows individual muscle twitch in a myoblast-injected wound. Note the rapid contraction and relaxation rates. The second panel shows that individual twitches began to superimpose with a stimulation frequency of 4 Hz, with a resulting potentiation in developed tension. The third panel shows that tetanus was induced with stimuli $\geq$ 7 Hz. Note the further increase in tension compared with the 4-Hz stimulation. Time to peak force in this preparation was $\sim$ 1 s, faster than was typically observed for the overall group. The fourth panel shows that no tension was developed at any voltage in a sham-injected wound. This tracing is representative of six wound strips from three sham-injected hearts. (*B*) Force–voltage relationship. Developed tension for individual twitches increased as stimuli increased from 30 to 100 V, indicating recruitment of additional myofibers. Data have been normalized to maximal developed tension and are presented as mean$\pm$SEM of eight wound strips from six hearts. (*C*) Fatigue test. Wounds containing myoblast grafts were subjected to a cardiac-like duty cycle, consisting of repeated episodes of 0.33 s of tetanus/0.67 s of rest, to mimic a heart rate of 60 beats/min. There was a 53% decrease in developed tension at the end of the 6-min test. Note that most of the diminution in force occurred during the first 3 min. Data represent mean$\pm$SEM of seven wound strips from five hearts.

myoblasts which had proliferated and subsequently differentiated. In the day 1 grafts, proliferating cells were present within the necrotic lesion, which could have represented either graft cells or macrophages. As mentioned above, none of the cells expressed MHC at this time, so it was not possible to determine which among these were myoblasts (versus transplanted fibroblasts or host macrophages). In the day 3 grafts, occasional BrdU-positive nuclei were identified within myosin-pos-

itive cells (Fig. 3 E). We observed a total of 12 such nuclei in three hearts. No attempt was made to quantify this low rate, but it was certainly < 1% of total nuclei in myosin-positive cells. Virtually no BrdU-positive nuclei were seen in myosin-positive cells at 1, 2, or 7 wk after transplantation (not shown). We conclude that myoblast proliferation occurs for at least 3 d after grafting, but by 1 wk virtually all cells have ceased replicating.

Contractile function of myoblast grafts. The contractile properties of 2-wk-old myoblast grafts were determined by attaching isolated wound strips to a tension myograph ex vivo. Virtually no spontaneous mechanical activity was detected, consistent with the paucity of cardiomyocytes histologically. Electrical stimulation caused muscle twitches in six of eight myoblast-engrafted hearts (Fig. 4 A, *first panel*); strips from the remaining two hearts may have been damaged during sample preparation, since skeletal muscle was present histologically. The grafts showed a stepwise increase in tension development as voltage was increased from 30 to ~ 100 V with a plateau thereafter (Fig. 4 B). This indicates that increasing voltage recruited additional myofibers to contract, implying that the graft myofibers are electrically insulated from one another. It should be noted that cardiac muscle does not increase contractile force with increasing voltage, since cardiocytes are coupled electrically via gap junctions.

Next, force–frequency relationships were determined. Using 120% of the voltage required for maximal tension development, the frequency of stimulation was increased incrementally from 0.5 to 10 Hz. Twitches began to superimpose at frequencies of 3–4 Hz, with a resulting increase in total developed tension (Fig. 4 A, *second panel*). Fully fused tetani were produced with 6–7 Hz stimulation (Fig. 4 A, *third panel*). Peak force during tetanus was 1.98±0.45 grams (mean SEM); after normalization to cross-sectional area the peak force was 0.72±0.14 grams/mm². The time to peak tetanic force averaged 2.3±0.3 s, although 90% of peak force was typically generated within 1.5 s. The time to half-relaxation after tetanus was 240±17 ms. It should be stressed that tetanus cannot be induced in cardiac muscle, due to the long refractory period of cardiocytes.

Finally, a fatigue test was performed to test the response of this muscle to a cardiac-like work load. The grafts were subjected to a duty cycle consisting of repeated 0.33 s of tetanic stimuli followed by 0.67 s of rest, mimicking a heart rate of 60 beats/min. The grafts showed a 32% decline in developed tension by 2 min and a 53% decline by the end of the 6-min test period (Fig. 4 C). No contractile activity could be elicited from six of seven wound strips from three injured hearts which received a sham injection of saline instead of myoblasts (Fig. 4 A, *fourth panel*). In one sham heart an adhesion had developed between the heart and chest wall, resulting in a small amount of intercostal muscle adhering to one of the two wound strips. In this preparation we detected a peak force of 0.04 grams/mm², < 2% of what was present in the myoblast-engrafted hearts.

Thus, the skeletal muscle grafts could be stimulated to contract ex vivo and could sustain a cardiac-like duty cycle over a 6-min test period. Furthermore, the grafts showed two physiological properties unique to skeletal muscle: recruitment of fibers with increasing voltage and the ability to sustain tetanic contraction. We do not know yet whether the grafts contract in vivo.

Discussion

The principal findings of this study are: (a) neonatal skeletal myoblasts can be grafted into an injured heart; (b) the engrafted myoblasts initially proliferate and then begin to form multinucleated myotubes by day 3; (c) the myotubes differentiate into mature myofibers, which initially have a phenotype similar to fast twitch muscle; (d) the myofibers develop characteristics of slow twitch muscle as the wound heals; (e) the new muscle may form satellite stem cells; and (f) the new muscle can be stimulated to contract ex vivo.

Strategies for muscle regeneration after myocardial injury. In principal, there are at least three strategies to induce muscle regeneration after myocardial infarction. First, the surrounding cardiac myocytes could be stimulated to migrate into the wound and proliferate to repair the defect. There is evidence that a limited amount of cell replication by adult cardiocytes occurs naturally after myocardial infarction in humans (17) and in rats (18, 19), but the response is clearly not adequate to repair the defect. The factors responsible for cell cycle arrest in cardiocytes are not well enough defined at present to begin exploring this as a therapy. (The interested reader is referred to references 20–23 for further information on this topic.)

A second strategy is to induce the cells of cardiac granulation tissue (the fibroblast-rich tissue of wound repair) to differentiate into muscle rather than forming a scar. There is not enough known about cardiac differentiation at present to attempt formation of new myocardium. However, much more is known about skeletal muscle determination. The discovery of myogenic determination genes (24, 25) has made it possible to induce a wide range of cultured cell types to differentiate into skeletal muscle. Recent studies from our group (26) and others (27) have shown that cells in cardiac granulation tissue can be induced to differentiate into skeletal muscle by transfection with the prototype myogenic determination gene, MyoD. In these early experiments, however, the frequency of muscle differentiation has been low after MyoD gene transfer. Until the frequency of myogenic conversion can be increased, it will be difficult to investigate the functional properties of the MyoD-induced skeletal muscle.

The third strategy for muscular repair of infarcts is to transplant either skeletal or cardiac myoblasts into the injured region. Studies by Koh et al. (3) and Soonpaa et al. (2) have demonstrated that fetal cardiocytes will form intercalated discs with host cardiocytes, including gap junctions and adherens junctions, when transplanted into normal hearts. No proliferation was detected in the grafted cardiocytes. Less information is available on grafting of cardiocytes into injured hearts. Our group (28) and others (29, 30) have preliminary data showing that neonatal rat or fetal human cardiocytes can be transplanted successfully into injured rat hearts. To our knowledge there is no information regarding proliferation of these grafts, nor are any functional data available. As discussed above, the principal limitation to this approach is the inability to induce cardiocytes to proliferate in culture. Until cardiocytes can replicate in vitro, or proliferation-competent cells can be induced reliably to differentiate into cardiocytes, cardiocyte grafting will not be feasible in humans.

In contrast to cardiocytes, proliferating skeletal muscle precursors are readily available, either as primary myoblasts in developing muscle or as satellite cells from quiescent muscle. In this study six rat pups yielded the myoblasts implanted into

27 injured hearts. In addition to their growth in culture, the myoblasts proliferated in vivo for several days after transplantation (Fig. 3 *E*). These properties have led us and several other groups to explore skeletal muscle grafting for cardiac repair. Koh et al. (7) demonstrated that the myogenic cell line C2C12 could be transplanted into the hearts of normal syngeneic mice, where the cells fused to form multinucleated myofibers. The same group also demonstrated that C2C12 cells stably transfected with a plasmid encoding active TGF-β could induce angiogenesis around the graft site (31). No coupling between the host cardiocytes and the grafted skeletal muscle was observed in either experiment.

Chiu et al. (8, 9) transplanted autologous satellite cells into cardiac freeze-thaw lesions in dogs. Comparable with our study, they also found that the grafts formed muscle cells within the healing lesion. In distinction to the current study, however, they hypothesized that their grafted skeletal muscle cells differentiated into cardiac muscle, via "milieu-dependent effects." The evidence for a cardiac phenotype was that some cells within the grafts had central rather than peripheral nuclei, and some cells contained refractile transverse structures under light microscopy interpreted to be intercalated discs. Although we observed some myofibers with persistent central nuclei in this study, as well as rare cells showing intermediate and gap junctions (Fig. 2, *C* and *D*), no intercalated discs were present by electron microscopy. More importantly, the grafted cells expressed skeletal muscle–specific proteins and failed to express the cardiac-specific isoform MHC-α up to 3 mo after transplantation. Thus, there clearly was no cardiac differentiation in this study.

Conversion of grafts from fast to slow twitch muscle. Although the skeletal muscle grafts expressed the fast fiber isoform of MHC at 1 and 2 wk, they expressed β-MHC, a marker for slow twitch fibers, at 7 wk and 3 mo. This indicates that the grafts were converting to slow twitch fibers. Conversion was apparently more rapid when the myoblasts were injected into wounds where healing had been allowed to progress for 1 wk, as opposed to immediately after injury. In the delayed transplantation model the grafts expressed β-MHC at 2 wk, while in the immediate transplantation model this protein was not detected until 7 wk. It is possible that the growth factors and cytokines present in the early wound delay myoblast differentiation and subsequent fiber type conversion.

Slow fibers exhibit several important differences from fast fibers, including a slower shortening velocity, use of oxidative phosphorylation for ATP production, a higher mitochondrial content, a higher myoglobin content, and a much greater resistance to fatigue (16, 32). An interesting parallel is that the latissimus dorsi muscle also undergoes fiber type switching when it is conditioned for dynamic cardiomyoplasty. Cardiomyoplasty is an experimental therapy for heart failure, where skeletal muscle is wrapped around the heart to serve as a ventricular assist device (33). Untrained latissimus dorsi is a mixed fiber type muscle which fatigues rapidly with repeated stimulation. When conditioned by repeated electrical stimulation for 6 wk before surgery, however, the latissimus dorsi converts entirely to slow twitch fibers and becomes fatigue resistant (6). Only the conditioned, slow twitch muscle is able to assist cardiac function. This parallel suggests the intriguing possibility that repeated electromechanical stimulation leads to activation of the slow fiber phenotype. Since we did not test whether the environment of the heart contributed to fiber type conversion,

additional experiments will be required to determine the mechanism. The fact that the grafts differentiated into slow twitch fibers suggests that they may be suited to perform a cardiac type work load.

Will skeletal muscle transplantation augment cardiac function? This study definitively showed that myoblast grafting can generate new contractile tissue. The skeletal muscle grafts exhibited characteristic twitches when stimulated ex vivo (Fig. 4 *A*) and showed recruitment of contractile units with increasing voltage (Fig. 4 *B*). Furthermore, tetanus could be induced with rapid stimulation (Fig. 4 *A*, *second* and *third panels*), and the grafts could perform a cardiac-like duty cycle for 6 min (Fig. 4 *C*). Peak force during tetanus averaged 0.7±0.1 grams/mm². Since the wound strips contained < 50% of the myofiber content of normal muscle, due to inclusion of scar tissue, the force can be normalized to at least 1.4 grams/mm² muscle. Adult mammalian muscle can generate 15–35 grams/mm² force at tetanus, depending on fiber type (14, 34). Thus, the 2-wk grafts generated ~ 4–10% of the predicted force for mature skeletal muscle. Several factors may cause a lower than predicted force, including the relative immaturity of the 2-wk myofibers, stretching of the immature extracellular matrix, poor cell matrix attachments, or misalignment of some fibers relative to the axis of the wound strip.

Although preliminary, these results are encouraging and suggest that more detailed studies of contractile function are warranted in skeletal myoblast-engrafted hearts. A critical question is whether the skeletal muscle grafts contract in vivo. To provide coordinated mechanical assistance, the grafted cells ideally should form electrical and mechanical junctions with the host myocardium. In our grafts the skeletal muscle cells were insulated from the remaining myocardium by scar tissue, so there was no opportunity for myofiber–cardiocyte coupling to occur. Koh et al. (7) transplanted C2C12 myoblasts into normal mouse hearts and observed no cell junctions between grafted myofibers and host cardiocytes by electron microscopy. Although proliferating myoblasts have been reported to synthesize both gap junction proteins (35) and *N*-cadherin (36, 37), these proteins are typically absent from adult skeletal myofibers. By electron microscopy we observed evidence both for intermediate and gap junction formation between skeletal myofibers 2 wk after grafting (Fig. 2, *C* and *D*). This finding was infrequent, however, and it is unknown whether such junctions would persist in longer term grafts. If skeletal muscle will not couple spontaneously with cardiac muscle, it is possible that such junctions could be induced by stably transfecting skeletal muscle cells with genes for cardiac junctional proteins. Another possibility is that skeletal muscle grafts could be electrically paced in synchrony with the cardiac cycle. Pacing would require sufficient voltage to activate all of the fibers, and currently it is unknown whether this would have a deleterious effect on the surrounding myocardium.

In the uninjured heart there is a complex fiber geometry, where the outer fibers run in the long axis, the midwall fibers run in the short axis, and the inner fibers again run in the long axis. This geometry is established during embryogenesis and is thought to be important for mechanical efficiency. In this study, the grafted myofibers were predominantly aligned with the short (transverse) axis of the heart. Alignment was noted as early as day 3, when myotube formation was prominent (Fig. 1 *C*). This is the same orientation that wound fibroblasts and collagen fibers acquire during wound healing, and it seems

likely that all are aligned by local mechanical forces. It is not known whether alignment with the heart's short axis will influence the ability of these myofibers to restore mechanical function after injury.

There are two aspects of skeletal muscle which theoretically could make it superior to cardiac muscle for infarct repair. First, skeletal muscle is much more resistant to ischemia than cardiac muscle. Skeletal muscle can withstand many hours of severe ischemia without becoming irreversibly injured, whereas in myocardium irreversible injury begins within 20 min (38). A second difference is that skeletal myoblast grafts might establish satellite cells. Satellite cells are the resident stem cells in skeletal muscle and proliferate in response to injury. Once activated, satellite cells can fuse with damaged myofibers or establish new myofibers to replace those lost to necrosis. We observed cells within 2-wk grafts which were morphologically consistent with satellite cells by electron microscopy (Fig. 3, E and F). Thus, it is possible that infarcts repaired with skeletal myoblasts might become more resistant to a subsequent episode of ischemia or might be able to replace myofibers damaged by ischemia.

Acknowledgments

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EVIDENCE APPENDIX

ITEM NO. 24

Caplan et al. publication in <u>Journal of Orthopaedic Research</u>, entitled, "Mesenchymal Stem Cells" cited by Appellant as Reference ACA in the Sixth Supplemental Information Disclosure filed February 21, 2006

Mesenchymal Stem Cells*

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Summary: Bone and cartilage formation in the embryo and repair and turnover in the adult involve the progeny of a small number of cells called mesenchymal stem cells. These cells divide, and their progeny become committed to a specific and distinctive phenotypic pathway, a lineage with discrete steps and, finally, end-stage cells involved with fabrication of a unique tissue type, e.g., cartilage or bone. Local cuing (extrinsic factors) and the genomic potential (intrinsic factors) interact at each lineage step to control the rate and characteristic phenotype of the cells in the emerging tissue. The study of these mesenchymal stem cells, whether isolated from embryos or adults, provides the basis for the emergence of a new therapeutic technology of self-cell repair. The isolation, mitotic expansion, and site-directed delivery of autologous stem cells can govern the rapid and specific repair of skeletal tissues. Key Words: Mesenchymal stem cells—Bone—Cartilage—Differentiation—Self-cell therapy—Skeletal tissue—Embryo—Adult.

THE CONCEPT

It is generally agreed that in an embryo a mesenchymal stem cell is a pluripotent progenitor cell which divides many times and whose progeny eventually gives rise to skeletal tissues: cartilage, bone, tendon, ligament, marrow stroma, connective tissue (Fig. 1). By definition, these stem cells are not governed by or limited to a fixed number of mitotic divisions. Their progeny are affected by a number of factors, however, as they become tracked into very specific developmental pathways in which both intrinsic and extrinsic factors combine to control the molecular and cellular pattern of expression that results in specific tissues that perform specific functions based on their molecular repertoire (9,11).

Indeed, the progression from stem cell to final end phenotype is marked by discrete stages with transit from one stage to the next dependent on local cuing from surrounding cells (paracrine regulation) as well as signals emitted by the cell itself and the reception of its own signaling (autocrine regulation) (10,57). The sum of these various intrinsic and extrinsic signals defines the developmental position of the cells. Although difficult to reconstruct on a cell culture dish, such "positional information" has been experimentally approached by studying embryonic cells in culture, cells that have the potential to differentiate into various phenotypes (7,9,11,15).

The concept of stem cells is now well established (21,60). Two systems serve as models for such a concept: First, *Caenorhabditus elegans* is a small worm whose entire developmental lineage map has been described (21); every cell found in the adult has been carefully tracked and its progenitor tree precisely established with every branch and sub-branch delineated. Second, and to be emphasized, the heamtopoietic cell lineage has been described with its several diverging pathways (21,52). It is now clear that each separate pathway and, indeed,
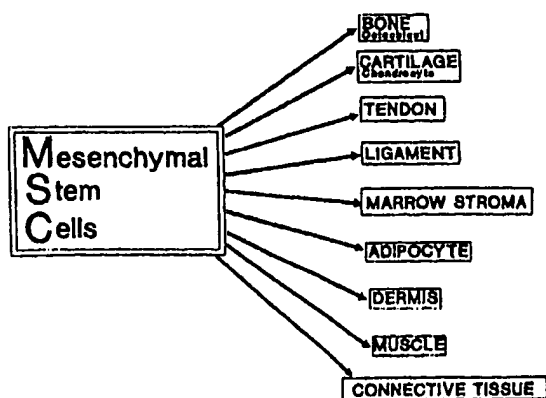
FIG. 1. Mesenchymal stem cell phenotypes. Mesenchymal stem cells are theoretically capable of differentiating through a series of separate and unique lineage transitions into a variety of end-stage phenotypes as shown.

progression through each separate stage within a discrete pathway is controlled by a balance of extrinsic and intrinsic macromolecules. Molecular biologists continue to isolate, clone, and express large amounts of these proteins, which allows use of cell culture systems to identify accurately the factor that controls progression to which stage and when (51,70). The challenge for skeletal biologists is to use the new information and new molecular tools to translate these advances into a better understanding of skeletal development, physiology, and repair.

EMBRYONIC MESENCHYMAL CELLS

The middle embryonic layer, the mesoderm, gives rise to all of the body's skeletal elements.* The term, mesenchyme, is derived from the Greek meaning "middle" (meso) "infusion" and refers to the ability of mesenchymatous cells to spread and migrate in early embryonic development between the ectodermal and endodermal layers. This characteristic migratory, space-filling ability is the key element of all wound repair in adult organisms involving mesenchymal cells in skin (dermis), bone (periosteum), or muscle (perimysium). Proteins that serve as chemoattractants, chemicals that specifically encourage this migratory activity to wound or developmental sites have been identified (24,32,59). The migratory activity of mesenchymal cells is complemented by their capacity to aggregate spe-

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* For the sake of clarity, I address only issues related to cartilage or bone, although the same general experimental approach and logic can be used for other mesenchymal tissues.

cifically to form unique developmental structures or, in adults, to form repair blastemas, which are then capable of responding to local cues and differentiating accordingly to achieve regenerative repair (10,11).

Chick Limb Cells

More than 20 years ago, my collaborators and I attempted to define experimentally the conditions and cues necessary to control the differentiation of embryonic mesenchymal cells into cartilage and bone (5,7,17). Both in vivo and in vitro studies were used, but development of cell cultures and the general approach of using cell cultures has provided the experiential basis for approaching the study of mesenchymal stem cells from adults. The system we developed was the culturing of stage 24 (day 4.5) embryonic chick limb mesenchymal cells under conditions that promoted differentiation of cartilage (chondrocytes) (5,7,13,20) and bone (osteoblasts) (42,65).

Chondrocytes

Our first experimental effort with embryonic chick limb mesenchymal cells was to focus on chondrocyte development, which we learned was controlled by the initial plating density (5,17), oxygen levels (14), or, as recently shown by other investigators, a variety of physical and chemical factors (53,58,61). The key factor in the conversion of a mesenchymal cell to a chondrocyte is maintaining the progenitor cell in a round, unspread conformation. This can be accomplished simply by plating the cells initially under very compact, high-density conditions: 5×10^6 embryonic stage-24 limb mesenchymal cells per 35-mm dish (5,17). Even in a simple, defined medium consisting of insulin, transferrin, bovine serum albumin (BSA), and hydrocortisone in Eagle's minimum essential medium (MEM), the differentiation of chondrocytes and their further development can be documented as long as the cells are initially seeded at high density (18,30).

The high-density, limb cell-derived chondrocyte in culture makes two cartilage-specific molecules in abundance: type II collagen (68) and a large chondroitin sulfate, keratan sulfate proteoglycan (CSPG) (13,18,20). By detailed chemical and physical characterization of the CSPG synthesized on each day of culture, we showed that the glycosaminoglycan chains are biosynthesized slightly differently with

time (Fig. 2). Peptide maps show that the newly synthesized core protein (26) is identical on each day of culture, whereas the chondroitin sulfate chains are synthesized progressively shorter (30,000 D on day 2 to 15,000 D on day 20) and the keratan sulfate chains are synthesized progressively larger (0 to 10,000 D) (13,20). This biosynthetic progression is exactly what has subsequently been shown to occur in the cartilages of embryonic, adult, and aging human (50) and bovine specimens (62).

That embryonic chondrocytes have an aging-dependent program of changing biosynthesis is further documented when cultured embryonic chick chondrocytes are transplanted in a fibrin-based delivery vehicle into defects at the articular surface of adult chickens (29). Such chondrocytes produce what appears to be appropriate cartilaginous matrix and have been followed >18 months. The resulting repair cartilage appears to integrate perfectly into the defect and to provide the animal with a healthy, normal articular surface. These experiments and others clearly establish the concept of repairing cartilage with embryonic or appropriate reparative cells.

Osteoblasts

Our initial success in studying emergence of chondrocytes and formation of cartilaginous tissue from cultures of limb mesenchymal cells encouraged us to study differentiation of osteoblasts and formation of bone as well. Our initial logic was that high-density conditions caused cartilage formation and that cartilage was the progenitor tissue of bone. (Some investigators have reported that cartilage

provides the scaffold for bone formation.) After 2 years of frustrating experimentation, we realized that when infrequent bone and osteoblasts could be identified, the bone had formed at a distance from cartilage and never on or in the cartilage (42). By carefully decreasing the initial cell density of limb mesenchymal cells to just below the density at which some mineralized cartilage could form (2 × 10^6 cells/35-mm dish), we observed numerous deposits of bone and abundant osteoblasts which, again, were clearly at some distance from cartilage (6,42,44). In addition, these osteoblasts exhibited the classic response to parathyroid hormone (PTH) of elevated cyclic AMP levels (71,72) and possessed a bone-specific alkaline phosphatase (43). These studies clearly indicated that embryonic chick limb mesenchymal cells were capable of differentiating into osteoblasts and that the culture conditions supporting optimum osteoblast emergence were different from the conditions optimum for chondrogenesis.

Mouse and Human Limb Cells

With regard to cartilage and bone, the properties of mouse and human limb mesenchymal cells in culture appear to be quite similar, if not identical (25,46). Likewise, cartilage and bone development in vivo are also quite comparable, with the major exception that embryonic cartilage of chick does not calcify whereas that of mammals always calcifies (16). The comparable developmental properties of aves, rodents, and humans encourages us to continue experimentation with animal cells as an approximation of better understanding of the properties of human cells and tissues.

FIG. 2. Proteoglycans synthesized by newly differentiated, mature, and senescent chondrocytes. With increasing age, chondrocytes synthesize proteoglycans that have smaller chondroitin sulfate chains and larger keratan sulfate chains (7,8,12,13,20).
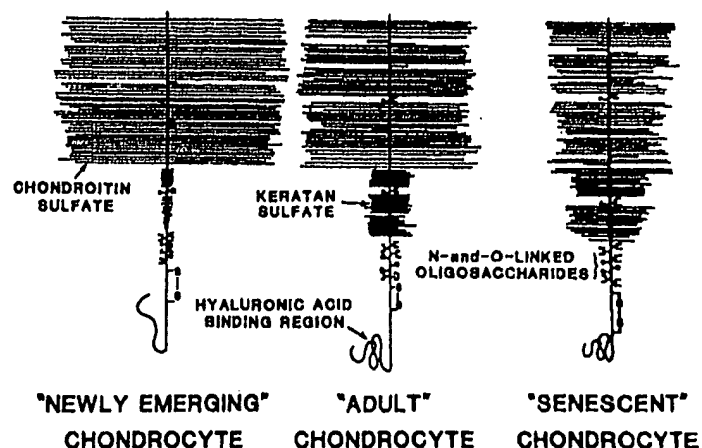
LINEAGE OF MESENCHYMAL CELLS

Cartilage

The important inference from the above discussion is that chondrocytes have a programmed (i.e., genetically dictated) sequence of changes in their end-stage expression (8,12). The differences in glycosaminoglycan chain lengths or chemistry are stable to cell culturing or metabolic perturbation. The control of these events is not known, but all experiments designed to slow this sequence of biosynthetic alterations or reverse them have failed. The inference is that a genomic mechanism somehow "tells time" and that this clock is hard-wired and unidirectional (8,12).

Such biosynthetic changes in articular cartilage are different from the lineage changes observed in adult growth plate or embryonic limb cartilage. A discrete set of expressional stages or lineage states, comprising dividing, maturing, and hypertrophic chondrocytes, is apparent in embryonic limb tissue, cell culture (13,58,61), and in the growth plate (19,28). Eventually, the hypertrophic cartilage in vivo is eroded by vascular, marrow, and phagocytic cells and replaced by bone. Each chondrocytic lineage state is uniquely different from its predecessor, as shown in Fig. 3. For example, hypertrophic chondrocytes synthesize a unique small collagen, type X, and a unique proteoglycan (54,55); neither of these molecules is synthesized by mature chon-

drocytes. In this particular circumstance, several factors are proposed to contribute to conversion of mature chondrocytes to hypertrophic chondrocytes (35); reversal of this process has not been reported.

Bone

We recently reviewed the major aspects of embryonic bone development. Figure 4 shows several important elements or rules governing this complex process (10,11,16). First, a discrete positioning of progenitor cells, stacked cells, existed in proximity to the developing bone (47). The stacked cells give rise to osteoblats in a discrete series of lineage steps (described below). The end stage or secretory osteoblast is positioned by its proximity to vasculature, with the "back" of the osteoblast to the capillary and osteoid deposited from the "front" of this highly oriented secretory cell (47,48). The vasculature is the orientor of osteogenesis and the osteoblast is the formative element. Cartilage is not replaced by bone, but is instead the target for vascular (marrow) replacement (48); in the early limb, the cartilage model exactly defines the eventual marrow cavity.

That a discrete series of individual lineage stages exists between the progenitor cells in the stacked cell layer and the secretory osteoblasts is now clear, as shown in Fig. 5. We recently isolated four monoclonal antibodies, SB1, 2, 3, and 5, which have helped provide evidence for an osteoblast lineage (3,4). Progenitor cells in the stacked cell layer and osteocytes do not interact with SB1, 2, or 3. Newly differentiated osteogenic cells react with SB1, but not with SB2 or 3, whereas fully secretory osteoblasts react with SB1, 2, and 3. A subpopulation of osteogenic cells reacts with SB2, but not SB3. Osteocytes react with OB7.3 of Nijweide and Mulder (38) or with our SB5, but not with SB1, 2, or 3. The lineage tree in Fig. 3 is based on these observations and not only establishes the existence of an osteoblastic lineage but suggests that osteocytes are derived directly from osteoblasts with SB1, 2, and 3 antigens that are suppressed as SB5 and OB7.3 are turned on. Experiments are now in progress to use these monoclonal antibodies to isolate representatives of each lineage stage so that studies can be conducted to identify the agents that promote the progression from one lineage stage to the next. Central to the thesis presented below is the existence of osteoprogenitor cells in the stacked cell layer, the future periosteum.

Chondrogenic PROGENITOR   I Type I Collagen, CSPG-M

↓

Chondroblast   I Type II Collagen, CSPG-H

↓

Chondrocyte I   I 148 Kd, link protein

↓

Chonrocyte II   I 100 Kd

↓

Hyportrophic Chondrocyte   I Type X collagen, 59 Kd, 1,25OH D₃R
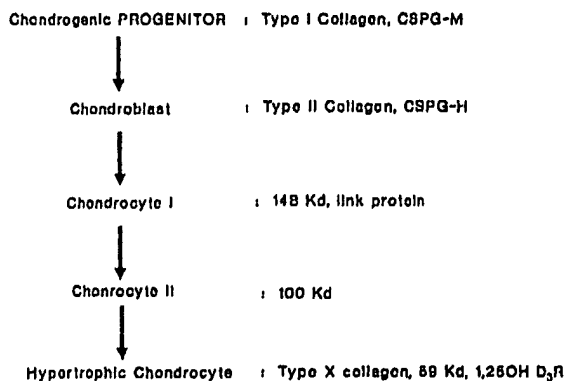
FIG. 3. Chondrogenic lineage. Based on the experiments of Solursh et al. (58,61) a hypothetical lineage map can be constructed to consist of at least five separate stages based on the changing biosynthesis of proteins (named or by molecular weight, K_d) or chondroitin sulfate proteoglycan (CSPG). The receptor for 1,25-dihydroxy Vitamin D_3 is represented as 1,25OHD₃R.

FIG. 4. Sequence of progressive in vivo bone development. Progressive repositioning of the vasculature from outside the stacked cell layer to a position in close approximation to the first layer of secretory osteoblasts responsible for formation of the first bony collar of the chick tibia (11,47,48). The osteoblast is oriented with its back toward the invading capillary and secretion of osteoid toward the cartilage core from the osteoblast's face. In this model, osteoblasts secrete osteoid in a direction away from vasculature (B), causing formation of a strut (C) and eventually forming the second layer of bone (D). These observations show that an intimate relationship exists between vasculature and newly forming bone.
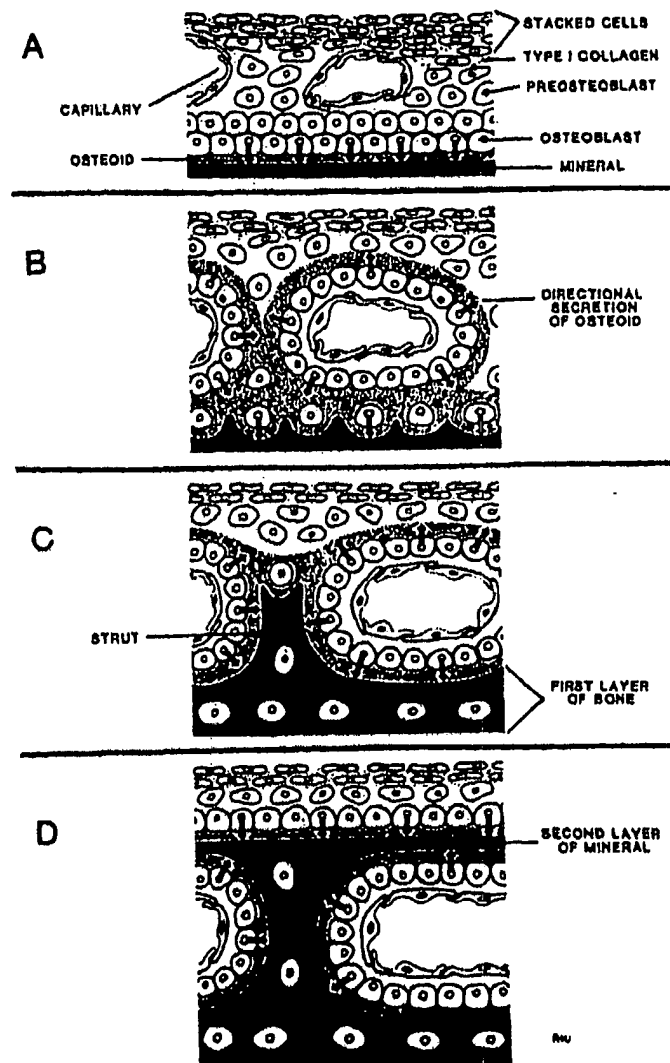
BIOACTIVE FACTORS IN BONE

From the earliest days of modern humans, bone has been recognized to have the powerful capacity to repair discontinuities (22). A variety of bioactive factors combine in a complex multicellular, multistep response in which reparative cells are specifically attracted to the repair site. These cells then aggregate, multiply, bridge the bone gap, and differentiate into chondrocytes or osteoblasts as controlled by the proximity to vasculature. Recently, an intensive research activity to identify and characterize these various bioactive factors was largely

successful (56,66,67,69). Our laboratory has described the purification of a protein factor, chondrogenic stimulating activity (CSA), which converts embryonic limb mesenchymal cells to chondrocytes (63,64). We are also attempting to purify a bone-derived chemoattractant for mesenchymal cells by using the now standard modified Boyden chamber (31,33).

Relevant to the thesis developed below, the identity and manipulation of the cells responding to bone-derived bioactive factors is directly related to successful bone repair. Such responding cells are present in the adult periosteum (36), dermis (49),

Osteo-progenitor   No probes currently available

|

Pre-Osteoblast   SB-1 (anti-Alkaline Phosphatase)-positive
                            Non-secretory, mitotic

|

Transitory 1 Osteoblast   SB-1-, SB-3-positive
                            Non-secretory, mitotic

|

Transitory 2 Osteoblast   SB-1-, SB-2-, SB-3-positive
                            Non-secretory, mitotic

|

Secretory Osteoblast   SB-1-, SB-2-, SB-3-positive
                            Secretory, non-dividing

|

Osteocytic Osteoblast   SB-2-, SB-5-positive, SB-1-, SB-3-negative
                            Secretory?, non-dividing

|

Osteocyte   SB-5-positive, SB-1-, SB-2-, SB-3-negative
                            Maintains bone physiology ?

Proposed osteoblastic differentiation ───────────────►

AlPase   ─────────────────────►
SB-1   ─────────────────────►
SB-3   ───────────────►
SB-2   ───────────────────►
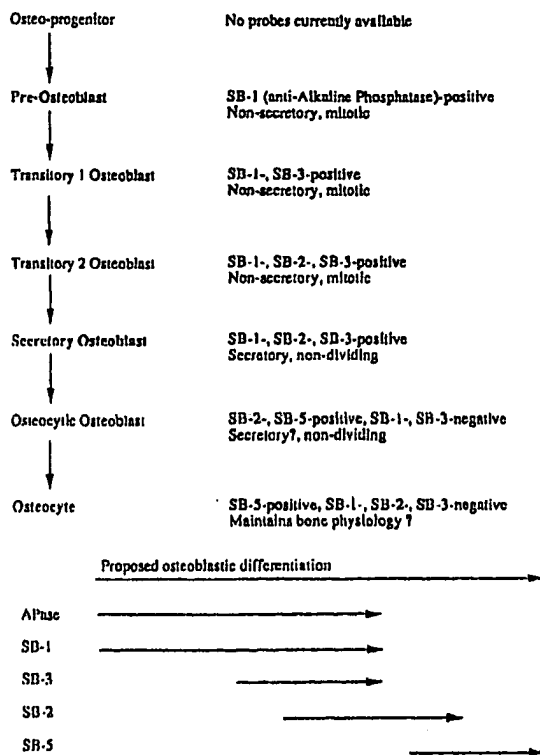SB-5   ───────────────────►

FIG. 5. Osteogenic cell lineage. Based on recent experimentation in which monoclonal antibodies were generated to cell surface antigens of osteogenic cells (3,4), a tentative lineage diagram reflects acquisition or loss of specific antigenic determinants. The characteristics of SB1, 2, and 3 were described previously (3); SB-5 (4) has been characterized and is similar to OB7.3 of Nijweide and Mulder (38). The individual lineage states are not weighted in terms of their prevalence or dwell-time; e.g., "transitory osteoblast 1" occurs rarely and cannot be recognized easily except at specific times and locations, whereas the "secretory osteoblast" is easily recognized and plentiful.

bone marrow (1,40,41,45), and connective tissue associated with muscle (34,37). One or all of these repositories are capable of forming bone when appropriately delivered bioactive factors are presented.

Alternately, when the responsive cells, stem cells, are placed in suitable delivery vehicles that can retain these cells while encouraging vascular invasion, bone can be observed to form. Recently, we used calcium phosphate porous ceramics in composite with marrow to encourage bone formation at both heterotopic and orthotopic sites (40,41). Whole disaggregated marrow cells in suspension are loaded into porous ceramic and transplanted to subcutaneous, intramuscular, or bone defect sites

in vivo. In 1–2 months, the few mesenchymal stem cells in the marrow have replicated massively and differentiated into osteoblasts. In the dead-end pores of the ceramic, which are devoid of vasculature, these stem cells differentiate into chondrocytes and form cartilage.

MESENCHYMAL STEM CELLS

From the above discussion several key facts are evident. First, embryonic mesenchymal stem cells in the limb which give rise to cartilage and bone in vivo can be manipulated in vitro. Second, these cells have a lineage progression of separate, individual steps, whether it be the chondrogenic or osteogenic pathway. Third, local cuing, sometimes involving highly potent protein factors, is responsible for providing positional information and causing lineage progression. Cell culture conditions have been refined to the extent that not only can these progressive events be studied in detail, but manipulation of the cells is also possible to provide control of tissue size and function.

Fourth, although chondrocytes and osteoblasts are derived from a common mesenchymal cell, the conditions for their initial differentiation and progression through the individual steps of their lineages are uniquely different. For example, osteogenesis is dependent on proximity to vasculature whereas chondrogenesis requires the complete absence of vasculature (7,10,11,16); osteogenesis is optimum at an initial cell culture seeding density in 35-mm dishes of 2×10^6 embryonic limb mesenchymal cells, whereas chondrogenesis is optimum at 5×10^6 cells (5,17,42).

Fifth, bone forms from mesenchymal stem cells in a cartilage-independent manner with vasculature providing a determinative discriminator between these two tissues; embryonic cartilage is not replaced by bone, but rather by vasculature and marrow (10,11,16). Sixth, we can demonstrate that three tissue sites are the repositories of mesenchymal stem cells: marrow (1,40,41,45), periosteum (36), and muscle connective tissue (34,37).

MARROW

Figure 6 outlines an assay to demonstrate that marrow contains mesenchymal stem cells capable of differentiation into cartilage and bone. Whole marrow is disrupted into single cells by passing it through needles of successively smaller sizes; the
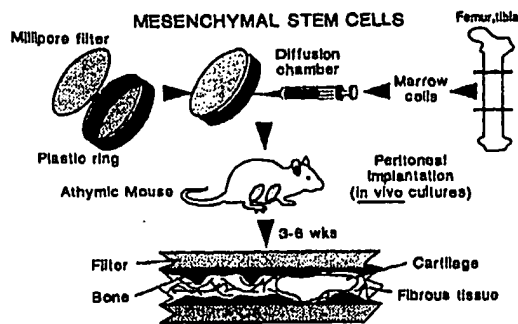
FIG. 6. Diffusion chamber assay in nude mice. Cell samples from marrow or other sources can be loaded into chambers composed of two Millipore filters glued to the edges of a plastic ring. These chambers are then implanted in the peritoneal cavity of athymic (nude) mice as a highly vascular in vivo incubation site. The filters prevent host cells from entering the chambers but permit rapid diffusion of nutrients and other factors into or out of the chamber. Histologic identification of two distinctive phenotypes, cartilage and bone, indicates that mesenchymal stem cells were present in the initial inoculum (1,2,45).

cells are counted, and 1–10 × 10^6 cells are placed in a small diffusion chamber (1,2,45). This chamber is of simple construction consisting of a small plastic ring onto which two Millipore filters have been glued. The filters allow body fluids (salts, nutrients, proteins, large protein complexes) to pass in and out of the chamber, but cells inside are not mixed with host cells, and tissues such as the vasculature are completely excluded. These chambers are implanted into the peritoneal cavity of an athymic (nude) mouse as an in vivo incubator, and they quickly become surrounded by host vasculature. Detailed studies have shown that the hematopoietic cells are eliminated, whereas mesenchymal cells vigorously divide and differentiate into cartilage in the middle of the chamber and bone at the filter interfaces closest to the enveloping vasculature (1,2,45). The presence of both cartilage and bone in the diffusion chamber has been compared to the presence of predominantly bone inside the highly vascularized pore regions of porous calcium phosphate ceramics loaded with marrow cells and implanted at heterotopic or orthotopic sites described above (40,41).

As a refinement of these experiments, we have been able to purify marrow mesenchymal cells by their differential adhesion to culture dishes and have successfully cultured cells through many passages (23). These cultured marrow mesenchymal cells from rat or chicken retain their capacity to differentiate into osteoblasts in ceramics through

such subculturing. Of importance is the demonstrated success of isolating marrow mesenchymal cells and mitotically expanding these cells with retention of their full developmental potency to differentiate into osteoblasts or chondrocytes.

Periosteum

Another repository for mesenchymal stem cells is the periosteum, a complex layer of cells that composes the outermost layer of long bone; we have termed the periosteum the stacked cell layer in developing embryos (1,16,47,48). This layer clearly responds to injury by rapidly expanding and forming woven bone; it also has cells capable of differentiating into chondrocytes when the periosteum is transplanted into an articular cartilage defect (39). In experimentation paralleling that described above for marrow mesenchymal cells,we have been successful in culturing and passaging periosteal cells (36). In porous ceramics implanted in nude mice, these cultured periosteal cells differentiate into osteoblasts (36). When the same cell preparation is injected into a subcutaneous site in a nude mouse, the cultured periosteal cells differentiate into both bone and cartilage (36). The important point is that culture-expanded periosteal cells retain their full developmental potency and can be manipulated to form two very complex and different tissues, bone or cartilage.

THE FUTURE: (SELF-CELL THERAPY)

Several important conceptual and technical advances have converged to allow us to consider the possibility of using a patient's own mesenchymal stem cells as starting material for tissue repair protocols. Mesenchymal stem cells must exist to maintain the living organisms, just as hematopoietic stem cells must exist to support both red and white blood cell turnover. Developmental biology has taught us that differentiated cells arise in a sequence of definitive cellular and molecular transitions, a lineage, from stem cell to end phenotype. Bone, for example, turns over; new osteoblasts arise, have a defined half-life, make new bone, and then die, to be replaced by other newly differentiating end-stage osteoblasts. Such osteoblasts must arise from stem cells; thus, a living organism must have repositories of stem cells.

Therefore, we might be able to isolate such human mesenchymal stem cells and place them in cell

culture, where we could mitotically expand their numbers. Eventually, if we had enough of these cells, we could reintroduce them into the original donor in a manner that guaranteed that they would massively differentiate into a specific tissue, such as cartilage or bone, at a transplantation or repair site. Immunorejection would not be a problem because the donor and host would be one and the same.

The first experimental step to test this idea is to determine if the animal-based technology described above can be modified to be used with human material. The first attempts at this have been highly encouraging. Recently, human marrow was introduced into diffusion chambers which were placed in nude mice; both cartilage and bone were eventually observed in the chamber (2). We recently cultured human marrow and isolated mesenchymal cells that were passaged, introduced into porous ceramics, and implanted subcutaneously in nude mice. In the pore regions of these highly vascularized composites, bone clearly formed in every sample of culture-expanded, marrow-derived mesenchymal cells tested (27). These preliminary experiments provide hope that the animal-based technology developed for mesenchymal cells from marrow or periosteum will be translatable to humans.

The concept of ex vivo manipulation of cells and their reimplantation into a donor is the basis for proposing self-cell therapy as a future possibility. Massive bone regeneration to fill gaps from tumor excision, regeneration of damaged articular cartilage, and maintenance of bone formation in the elderly at risk for osteoporosis are clinical protocols that require large numbers of the appropriate reparative skeletal cells. The patient's own mesenchymal stem cells may prove to be the basis of a new, cell-based treatment plan requiring the merging of molecular biology to produce specific bioactive factors, cell biology to develop ex vivo manipulation regimens, and surgeons able to implant cells capable of repairing skeletal defects by the regeneration process.

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EVIDENCE APPENDIX

ITEM NO. 25

Strauer et al. 2002 publication in <u>Circulation</u> entitled, "Repair of Infarcted Myocardium by Autologous Intracoronary Mononuclear Bone Marrow Cell Transplantation in Humans" cited by Appellant as Reference ABQ in the Third Supplemental Information Disclosure Statement filed May 27, 2003

Repair of Infarcted Myocardium by Autologous Intracoronary Mononuclear Bone Marrow Cell Transplantation in Humans

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Background—Experimental data suggest that bone marrow–derived cells may contribute to the healing of myocardial infarction (MI). For this reason, we analyzed 10 patients who were treated by intracoronary transplantation of autologous, mononuclear bone marrow cells (BMCs) in addition to standard therapy after MI.

Methods and Results—After standard therapy for acute MI, 10 patients were transplanted with autologous mononuclear BMCs via a balloon catheter placed into the infarct-related artery during balloon dilatation (percutaneous transluminal coronary angioplasty). Another 10 patients with acute MI were treated by standard therapy alone. After 3 months of follow-up, the infarct region (determined by left ventriculography) had decreased significantly within the cell therapy group (from 30 ± 13 to $12\pm7\%$, $P=0.005$) and was also significantly smaller compared with the standard therapy group ($P=0.04$). Likewise, infarction wall movement velocity increased significantly only in the cell therapy group (from 2.0 ± 1.1 to 4.0 ± 2.6 cm/s, $P=0.028$). Further cardiac examinations (dobutamine stress echocardiography, radionuclide ventriculography, and catheterization of the right heart) were performed for the cell therapy group and showed significant improvement in stroke volume index, left ventricular end-systolic volume and contractility (ratio of systolic pressure and end-systolic volume), and myocardial perfusion of the infarct region.

Conclusions—These results demonstrate for the first time that selective intracoronary transplantation of autologous, mononuclear BMCs is safe and seems to be effective under clinical conditions. The marked therapeutic effect may be attributed to BMC-associated myocardial regeneration and neovascularization. (*Circulation.* 2002;106:1913-1918.)

Key Words: myocardial infarction ■ cell transplantation, intracoronary ■ angiogenesis ■ bone marrow ■ myogenesis

Remodeling of the left ventricle after myocardial infarction (MI) represents a major cause of infarct-related heart failure and death. This process depends on acute and chronic transformation of both the necrotic infarct region and the non-necrotic, peri-infarct tissue.[1,2] Despite application of pharmacotherapeutics and mechanical interventions, the cardiomyocytes lost during MI cannot be regenerated. The recent finding that a small population of cardiac muscle cells is able to replicate itself is encouraging but is still consistent with the concept that such regeneration is restricted to viable myocardium.[3]

In animal experiments, attempts to replace the necrotic zone by transplanting other cells (eg, fetal cardiomyocytes or skeletal myoblasts) have invariably succeeded in reconstituting heart muscle structures, ie, myocardium and coronary vessels. However, these cells fail to integrate structurally and do not display characteristic physiological functions.[4-7] Another approach to reverse myocardial remodeling is to repair myocardial tissue by using bone marrow–derived cells. Bone

marrow contains multipotent adult stem cells that show a high capacity for differentiation.[8-10] Experimental studies have shown that bone marrow cells (BMCs) are capable of regenerating infarcted myocardium and inducing myogenesis and angiogenesis; this leads in turn to amelioration of cardiac function in mice and pigs.[11-14] However, procedures based on this phenomenon remain largely uninvestigated in a human clinical setting.

An investigation of one patient receiving autologous skeletal myoblasts into a postinfarction scar during coronary artery bypass grafting revealed improvement of contraction and viability 5 months afterward.[15] Autologous mononuclear BMCs transplanted in a similar surgical setting showed long-term improvement of myocardial perfusion in 3 of 5 patients and no change in 2 patients.[16] However, such studies entail a surgical approach and are therefore associated with well-known perioperative risks. Moreover, this surgical procedure cannot be used with MI. We therefore looked for a nonsurgical, safer mode for transplanting autologous cells

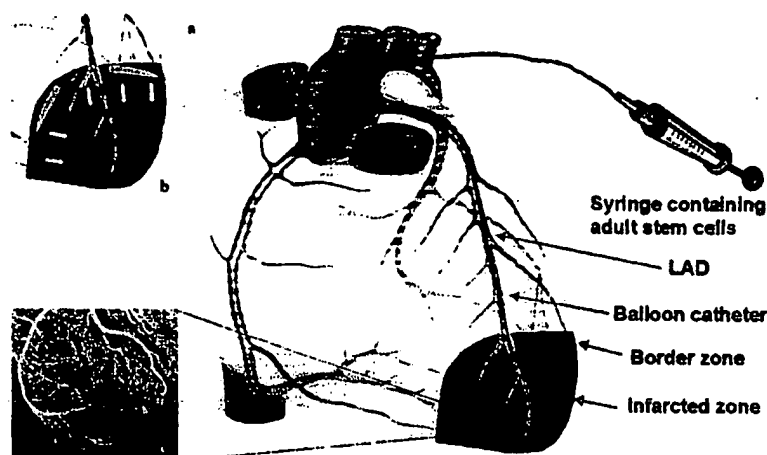DOI: 10.1161/01.CIR.0000034046.87607.1C

Figure 1. Procedure of cell transplantation into infarcted myocardium in humans. a, The balloon catheter enters the infarct-related artery and is placed above the border zone of the infarction. It is then inflated and the cell suspension is infused at high pressure under stop-flow conditions. b, In this way, cells are transplanted into the infarcted zone via the infarct-related vasculature (red dots). Cells infiltrate the infarcted zone. Blue and white arrows suggest the possible route of migration. c, A supply of blood flow exists within the infarcted zone.[35] The cells are therefore able to reach both the border and the infarcted zone.

into postinfarction tissue. A pilot study from our group demonstrated that intracoronary transplantation of autologous mononuclear BMCs 6 days after MI was associated with a marked decrease in infarct area and an increase in left ventricular (LV) function after 3 and 6 months of follow-up.[17] To confirm these results and validate this promising new therapy for MI, we established a clinical trial involving 20 patients for comparing the safety and bioefficacy of autologous BMC transplantation. All 20 patients underwent standard therapy, and 10 patients received additional intracoronary cell transplantation. All 20 patients were followed up for 3 months.

Methods

Patient Population

All 20 patients had suffered transmural infarction according to World Health Organization criteria with the involvement of the left anterior descending coronary artery (n=4), left circumflex coronary artery (n=3), or right coronary artery (n=13). Mean duration of infarct pain was 12±10 hours before invasive diagnostics and therapy. Patients had to be <70 years old and were excluded if one of the following criteria were met: screening >72 hours after infarction, cardiac shock, severe comorbidity, alcohol or drug dependency, or excessive travel distance to the study center.

After right and left heart catheterization, coronary angiography, and left ventriculography, mechanical treatment was initiated with recanalization of the infarct-related artery by balloon angioplasty (n=20) and subsequent stent implantation (n=19). All patients were monitored in our intensive care unit, and no arrhythmogenic events or hemodynamic impairments were recorded in either patient group.

All 20 patients were briefed in detail about the procedure of BMC transplantation. Informed consent was obtained from 10 patients, who formed the cell therapy group, whereas 10 patients who refused additional cell therapy served as controls. The local ethics committee of the Heinrich-Heine-University, Düsseldorf, approved the study protocol. All procedures conformed to institutional guidelines.

Before taking part in rehabilitation programs, all patients left the hospital with standard medication consisting of acetylsalicylic acid, an ACE inhibitor, a β-blocker, and a statin.

Bone Marrow Aspiration, Isolation, and Cultivation

Seven (±2) days after acute coronary angiography, bone marrow (≈40 mL) was aspirated under local anesthesia from ilium of cell therapy patients (n=10). Mononuclear BMCs were isolated by Ficoll density separation on Lymphocyte Separation Medium (BioWhittaker) before the erythrocytes were lysed with H_2O. For overnight

cultivation, 1×10^6 BMCs/mL were placed in Teflon bags (Vuelife, Cell Genix) and cultivated in X-Vivo 15 Medium (BioWhittaker) supplemented with 2% heat-inactivated autologous plasma. The next day, BMCs were harvested and washed 3 times with heparinized saline before final resuspension in heparinized saline. Viability was 93±3%. Heparinization and filtration (cell strainer, FALCON) was carried out to prevent cell clotting and microembolization during intracoronary transplantation. The mean number of mononuclear cells harvested after overnight culture was 2.8×10^7; this consisted of 0.65±0.4% AC133-positive cells and 2.1±0.28% CD34-positive cells. All microbiological tests of the clinically used cell preparations proved negative. As a viability and quality ex vivo control, 1×10^5 cells grown in HS100 medium (Stem Cell Technology) were found to be able to generate mesenchymal cells in culture.

Intracoronary Transplantation of BMCs

Five to nine days after onset of acute infarction, cells were directly transplanted into the infarcted zone (Figure 1). This was accomplished with the use of a balloon catheter, which was placed within the infarct-related artery. After exact positioning of the balloon at the site of the former infarct-vessel occlusion, percutaneous transluminal coronary angioplasty (PTCA) was performed 6 to 7 times for 2 to 4 minutes each. During this time, intracoronary cell transplantation via the balloon catheter was performed, using 6 to 7 fractional high-pressure infusions of 2 to 3 mL cell suspension, each of which contained 1.5 to 4×10^6 mononuclear cells. PTCA thoroughly prevented the backflow of cells and at the same time produced a stop-flow beyond the site of the balloon inflation to facilitate high-pressure infusion of cells into the infarcted zone. Thus, prolonged contact time for cellular migration was allowed.[18]

Functional Assessment of Hemodynamics

After 3 months, all 20 patients were followed up by left heart catheterization, left ventriculography, and coronary angiography. Ejection fraction, infarct region, and regional wall movement of the infarcted zone during ejection were determined by left ventriculography. Ejection fraction was measured with Quantcor software (Siemens). To quantify infarction wall movement velocity, 5 axes were placed perpendicular to the long axis in the main akinetic or dyskinetic segment of the ventricular wall. Relative systolic and diastolic lengths were measured, and the mean difference was divided by the systolic duration (in seconds). To quantify the infarct region, the centerline method according to Sheehan was used.[19] All hemodynamic investigations were obtained by two independent observers.

In the cell therapy group before and 3 months after cell transplantation, additional examinations for measuring hemodynamics and myocardial perfusion included dobutamine stress echocardiography, radionuclide ventriculography, catheterization of the right heart, and

TABLE 1.   Baseline Characteristics of the Patients

Clinical Data	Cell Therapy	Standard Therapy	P
Characteristics			
No. of patients	10	10	...
Age, y	49±10	50±6	NS
Sex	Male	Male	...
Onset of infarction before angioplasty, h	10±8	13±11	NS
Coronary angiography			
No. of diseased vessels	1.7±0.9	2.1±0.7	NS
No. of patients with LAD/LCX/RCA as the affected vessel	4/1/5	0/2/8	...
No. of patients with stent implantation	9	10	...
Laboratory parameters			
Creatinine kinase, U/L	1138±1170	1308±1187	NS
Creatinine kinase-MB, U/L	106±72	124±92	NS
Bone marrow puncture after angioplasty, d	7±2	...	...
Mononuclear bone marrow cells, n ($\times 10^7$)	2.8±2.2	...	...

Values are mean±SD or number of patients.
NS indicates not significant; LAD, left anterior descending coronary artery; LCX, left circumflex coronary artery; and RCA, right coronary artery.

stress-redistribution-reinjection [201]thallium scintigraphy. The contractility index P_{sys}/ESV was calculated by dividing LV systolic pressure (P_{sys}) by end-systolic volume (ESV). Perfusion defect was calculated by scintigraphic bull's-eye technique. Each examination was performed according to standard protocols.

There were no complications or side effects determined in any patient throughout the diagnostic or therapeutic procedure or within the 3-month follow-up period.

Statistical Analysis

All data are presented as mean±SD. Statistical significance was accepted when P was <0.05. Discrete variables were compared as rates, and comparisons were made by χ^2 analysis. Intra-individual comparison of baseline versus follow-up continuous variables was performed with a paired *t* test. Comparison of nonparametric data between the two groups was performed with Wilcoxon test and Mann-Whitney test. Statistical analysis was performed with SPSS for Windows (version 10.1).

Results

Clinical data between the two groups did not differ significantly. The range of creatinine kinase levels was slightly but not significantly higher in the standard therapy group than it was in the cell therapy group (Table 1).

Comparison of the 2 groups 3 months after cell or standard therapy showed several significant differences in LV dynamics, according to the global and regional analysis of left ventriculogram. The infarct region as a percentage of hypokinetic, akinetic, or dyskinetic segments of the circumference of the left ventricle decreased significantly in the cell therapy group (from 30±13 to 12±7%, P=0.005). It was also significantly smaller compared with the standard therapy group after 3 months (P=0.04). Within the standard therapy group, only a statistically nonsignificant decrease from 25±8 to 20±11% could be seen. Wall movement velocity over the infarct region rose significantly in the cell therapy group (from 2.0±1.1 to 4.0±2.6 cm/s, P=0.028) but not in the standard therapy group (from 1.8±1.3 to 2.3±1.6 cm/s, P=NS). No significant difference was observed between the

two groups. Ejection fraction increased in both groups, albeit nonsignificantly (from 57±8 to 62±10% in the cell therapy group and from 60±7 to 64±7% in the standard therapy group) (Table 2).

Further significant improvement could also be seen on additional analysis of the cell therapy group alone. Perfusion defect was considerably decreased by 26% in the cell therapy group (from 174±99 to 128±71 cm², P=0.016, assessed by [201]thallium scintigraphy) (Figure 2). Parallel to the reduction in perfusion defect, improvement (Table 3) could also be seen in:

(1) Cardiac function, as revealed by increase in stroke volume index (from 49±7 to 56±7 mL/m², P=0.010) and ejection fraction (from 51±14 to 53±13%, P=NS).

(2) Cardiac geometry, as shown by decreases in both end-diastolic (from 158±20 to 143±30 mL, P=NS) and end-systolic volume (from 82±26 to 67±21 mL, P=0.011). Radionuclide ventriculography was used to acquire the data.

(3) Contractility as evaluated by an increase in the velocity of circumferential fiber shortening (from 20.5±4.2 to 24.4±7.7 mm/s, P=NS, assessed by stress echocardiography) and by a marked increase in the ratio of systolic pressure to end-systolic volume (from 1.81±1.44 to 2.27±1.72 mm Hg/mL, P=0.005).

Discussion

The present report describes the first clinical trial of intracoronary, autologous, mononuclear BMC transplantation for improving heart function and myocardial perfusion in patients after acute MI. The results demonstrate that transplanted autologous BMCs may lead to repair of infarcted tissue when applied during the immediate postinfarction period. These results also show that the intracoronary approach of BMC transplantation seems to represent a novel

TABLE 2. Comparison of Cell Therapy and Standard Therapy Groups

	Cell Therapy	Standard Therapy	*P*
No. of patients	10	10	...
Infarct region as functional defect			
Hypokinetic, akinetic, or dyskinetic region at 0 mo, %	30±13	25±8	NS
Hypokinetic, akinetic, or dyskinetic region at 3 mo, %	12±7	20±11	0.04
P	0.005	NS	...
Contractility indices			
Infarction wall movement velocity at 0 mo, cm/s	2.0±1.1	1.8±1.3	NS
Infarction wall movement velocity at 3 mo, cm/s	4.0±2.6	2.3±1.6	NS
P	0.028	NS	...
Hemodynamic data			
LV ejection fraction at 0 mo, %	57±8	60±7	NS
LV ejection fraction at 3 mo, %	62±10	64±7	NS
P	NS	NS	...

NS indicates not significant; 0 mo, zero months, which means the time of infarction; 3 mo, 3 months, which means the time of the follow-up examinations. All data were obtained according to analysis of left ventriculogram.

and effective therapeutic procedure for concentrating and/or depositing infused cells within the region of interest.

Neogenesis of both cardiomyocytes and coronary capillaries with some functional improvement has been shown recently by several investigators using bone marrow–derived cells in experimental infarction.[11–14,18,20–23] Moreover, transendothelial migration from the coronary capillaries and incorporation of cells into heart muscle has been observed experimentally.[3,12,24–26] Until now, clinical data only existed for the cell therapy of surgically treated chronic ischemic heart disease.[15,16] Our aim was to transform the encouraging results from animal models to a safe clinical setting. The most crucial questions we had to address while designing and
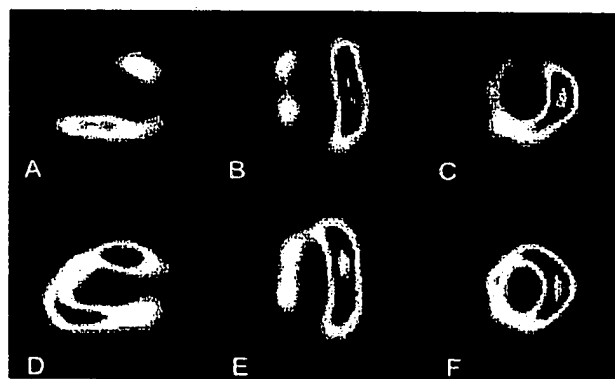

Figure 2. Improved myocardial perfusion of infarcted anterior wall 3 months after intracoronary cell transplantation subsequent to an acute anterior wall infarction detected by [201]thallium scintigraphy. The images on the left (A, D, sagittal) and in the middle (B, E) show the long axis, whereas those on the right (C, F, frontal) show the short axis of the heart. Initially the anterior wall, with green-colored apical and anterior regions, had reduced myocardial perfusion (A, B, C). Three months after cell transplantation the same anterior wall, now yellow in color, revealed a significant improvement in myocardial perfusion (D, E, F). All illustrations depict the exercise phase.

realizing this trial were: (1) What cell population should we deliver? (2) Which application method is the most efficient? (3) When should the cells be transplanted?

In recent years, several laboratories have shown that environmentally dictated changes of fate (transdetermination) are not restricted to stem cells but may also involve progenitor cells at different steps of a given differentiation pathway (transdifferentiation). Moreover, mesenchymal stem cells may represent an ideal cell source for treating different diseases.[27] Adult, mononuclear BMCs contain such stem and progenitor cells (≤1%), eg, mesodermal progenitor cells, hematopoietic progenitor cells, and endothelial progenitor cells. In several animal infarction models it has been shown that: (1) Bone marrow hemangioblasts contribute to the formation of new vessels; (2) bone marrow hematopoietic stem cells differentiate into cardiomyocytes, endothelium,

TABLE 3. Cardiac Function Analysis at 3-Month Follow-Up

	Before Cell Therapy	3 Months After Cell Therapy	*P*
No. of patients	10	10	...
Hemodynamic data			
LV ejection fraction, %	51±14	53±13	NS
Stroke volume index, mL/m²	49±7	56±7	0.010
Cardiac geometry			
LV end-diastolic volume, mL	158±20	143±30	NS
LV end-systolic volume, mL	82±26	67±21	0.011
Contractility indices			
Circumferential fiber shortening, mm/s	20.5±4.2	24.4±7.7	NS
P$_{mx}$/ESV, mm Hg/mL	1.81±1.44	2.27±1.72	0.005
Infarct region as perfusion defect			
[201]Thallium scintigraphy, cm²	174±99	128±71	0.016

NS indicates not significant.

and smooth muscle cells[8-13]; (3) BMCs give rise to mesodermal progenitor cells that differentiate to endothelial cells[28]; and (4) endothelial progenitors can transdifferentiate into beating cardiomyocytes.[29] Thus, several different fractions of mononuclear BMCs may contribute to the regeneration of necrotic myocardium and vessels. In order to utilize this large and perhaps heterogeneous regenerative potential, we decided to use all mononuclear cells from the bone marrow aspirate as a whole, rather than a subpopulation. No further expansion was performed because experimental data have revealed a dramatic decline in the homing capacity of in vitro amplified hematopoietic stem or progenitor cells.[30]

The second question was how to deliver the cells most efficiently. When given intravenously, only a very small fraction of infused cells can reach the infarct region after the following injection: assuming a normal coronary blood flow of 80 mL/min per 100 g of LV weight, a quantity of 160 mL per left ventricle (assuming a regular LV mass of ≈200 g) will flow per minute.[31,32] This corresponds to only about 3% of cardiac output (assuming a cardiac output of 5000 mL/min).[31] Therefore, intravenous application would require many circulation passages to enable infused cells to come into contact with the infarct-related artery. Throughout this long circulation and recirculation time, homing of cells to other organs could considerably reduce the numbers of cells dedicated to cell repair in the infarcted zone. Thus, supplying the entire complement of cells by intracoronary administration obviously seems to be advantageous for the tissue repair of infarcted heart muscle and may also be superior to intraventricular injection,[33] because all cells are able to flow through the infarcted and peri-infarcted tissue during the immediate first passage. Accordingly, by this intracoronary procedure the infarct tissue and the peri-infarct zone can be enriched with the maximum available amount of cells at all times.

As stem cells differentiate into more mature types of progenitor cells, it is thought that a special microenvironment in so-called niches regulates cell activity by providing specific combinations of cytokines and by establishing direct cellular contact. For successful long-term engraftment, at least some stem cells have to reach their niches, a process referred to as homing. Mouse experiments have shown that significant numbers of BMCs appear in liver, spleen, and bone marrow after intravenous injection.[34] To offer the BMCs the best chance of finding their niche within the myocardium, a selective intracoronary delivery route was chosen. Presumably, therefore, fewer cells were lost by extraction toward organs of secondary interest by this first pass–like effect. To facilitate transendothelial passage and migration into the infarcted zone, cells were infused by high-pressure injection directly into the necrotic area, and the balloon was kept inflated for 2 to 3 minutes; the cells were not washed away immediately under these conditions.

The time point for delivery was chosen as 7 to 8 days after infarction onset for the following reasons:

(1) In dogs, infarcted territory becomes rich in capillaries and contains enlarged, pericyte-poor "mother vessels" and endothelial bridges 7 days after myocardial ischemia and reperfusion. Twenty-eight days later, a significant muscular vessel wall has already formed.[35] Thus, with such timing, cells may be able to reach the worst

damaged parts and at the same time salvage tissue. Transendothelial cell migration may also be enhanced because an adequate muscular coat is not yet formed.

(2) Until now, only one animal study has attempted to determine the optimum time for cardiomyocyte transplantation to maximize myocardial function after LV injury. Adult rat hearts were cryoinjured and fetal rat cardiomyocytes were transplanted immediately, 2 weeks later, and 4 weeks later. The authors discussed the inflammatory process, which is strongest in the first days after infarction, as being responsible for the negative results after immediate cell transplantation, and they assumed that the best results seen after 2 weeks may have been due to transplantation before scar expansion.[36] Until now, however, no systematic experiments have been performed with BMCs to correlate the results of transplantation with the length of such a time delay.

(3) Another important variable is the inflammatory response in MI, which seems to be a superbly orchestrated interaction of cells, cytokines, growth factors and extracellular matrix proteins mediating myocardial repair. In the first 48 hours, debridement and formation of a fibrin-based provisional matrix predominates before a healing phase ensues.[37-40] Moreover, vascular endothelial growth factor is at its peak concentration 7 days after MI, and the decline of adhesion molecules (intercellular adhesion molecules, vascular cell adhesion molecules) does not take place before days 3 to 4 after MI. We assumed that transplantation of mononuclear BMCs within the "hot" phase of post-MI inflammation might lead them to take part in the inflammation cascade rather than the formation of functional myocardium and vessels.

Taking all of this into account, we can conclude that cell transplantation within the first 5 days after acute infarction is not possible for logistical reasons and is not advisable because of the inflammatory process. On the other hand, transplantation 2 weeks after infarction scar formation seems to reduce the benefit of cell transplantation. Although the ideal time point for transplantation remains to be defined, it is most likely between days 7 and 14 after the onset of MI, as in the present study.

This trial was designed as a phase I safety and feasibility trial, meaning that no control group is necessarily required. However, to validate the results, we correlated them with those obtained from 10 patients who refused to get additional cell therapy and thus received standard therapy alone. We are aware of the fact that such a comparison does not reach the power of a randomly allocated, blinded control group. However, the significant improvement with regard to infarct region, hemodynamics (stroke volume index), cardiac geometry (LV end-systolic volume), and contractility (P_{sys}/ESV and infarction wall movement velocity) did confirm a positive effect of the additional cell therapy because the changes observed in the standard therapy group failed to reach significance.

Another important factor for interpreting the results is time interval between onset of symptoms and revascularization of the infarct-related artery by angioplasty; this represents a crucial determinant of LV recovery. For patients with acute MI, it has

been shown that if the time interval is >4 hours, no significant changes in ejection fraction, regional wall motion, or ESV are observed after 6-month follow-up by echocardiography and angiography.[41] None of our 20 patients was treated by angioplasty within 4 hours after onset of symptoms. Our average time interval was 12±10 hours. Thus, PTCA-induced improvement of LV function can be nearly excluded; indeed, the only mild and nonsignificant changes within the standard therapy group are consistent with the above-mentioned data.[41] In contrast, the cell therapy group showed considerable and significant improvement in the same parameters, which may be attributed to BMC-mediated coronary angioneogenesis and cardiomyoneogenesis.

These results show that transplantation of autologous BMCs, as well as the intracoronary approach, represent a novel and effective therapeutic procedure for the repair of infarcted myocardium. For this method of therapy, no ethical problems exist, and no side effects were observed at any point of time. The therapeutic benefit for the patient's heart seems to prevail. However, further experimental studies, controlled prospective clinical trials, and variations of cell preparations are required to define the role of this new approach for the therapy of acute MI in humans.

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EVIDENCE APPENDIX

ITEM NO. 26

Kornowski U.S. Patent No. 7,097,832 cited by Appellant as Exhibit B in the Response filed November 28, 2007

US007097832B1

(12) United States Patent
Kornowski et al.

(10) Patent No.: **US 7,097,832 B1**

(45) Date of Patent: **Aug. 29, 2006**

(54) **INTRAMYOCARDIAL INJECTION OF AUTOLOGOUS BONE MARROW**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/868,411**

(22) PCT Filed: **Mar. 30, 2000**

(86) PCT No.: **PCT/US00/08353**

§ 371 (c)(1),
(2), (4) Date: **Jun. 14, 2001**

(87) PCT Pub. No.: **WO00/57922**

PCT Pub. Date: **Oct. 5, 2000**

Related U.S. Application Data

(60) Provisional application No. 60/126,800, filed on Mar. 30, 1999, provisional application No. 60/138,379, filed on Jun. 9, 1999.

(51) Int. Cl.
A01N 63/00 (2006.01)
C12N 5/00 (2006.01)
C12N 5/06 (2006.01)
(52) U.S. Cl. 424/93.7; 435/384; 435/372
(58) Field of Classification Search 424/94.1, 424/577; 514/2, 21; 435/325
See application file for complete search history.

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(57) **ABSTRACT**

A method of treating cardiac or myocardial conditions comprises the administration of an effective amount of autologous bone marrow. The bone marrow may optionally be stimulated and/or administered in combination with a pharmaceutical drug, protein, gene or other factor or therapy that may enhance bone marrow production of angiogenic growth factors and/or promote endothelial cell proliferation or migration or blood vessel formation.

15 Claims, 2 Drawing Sheets

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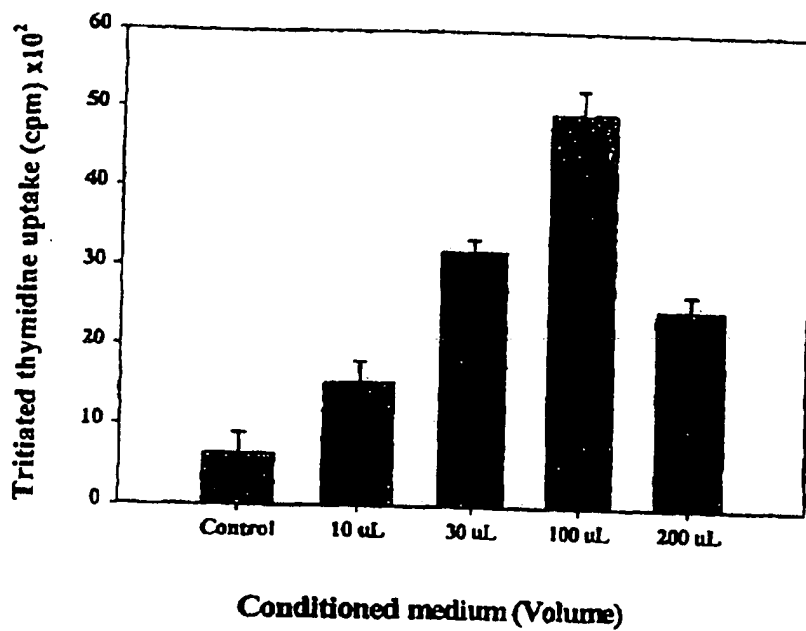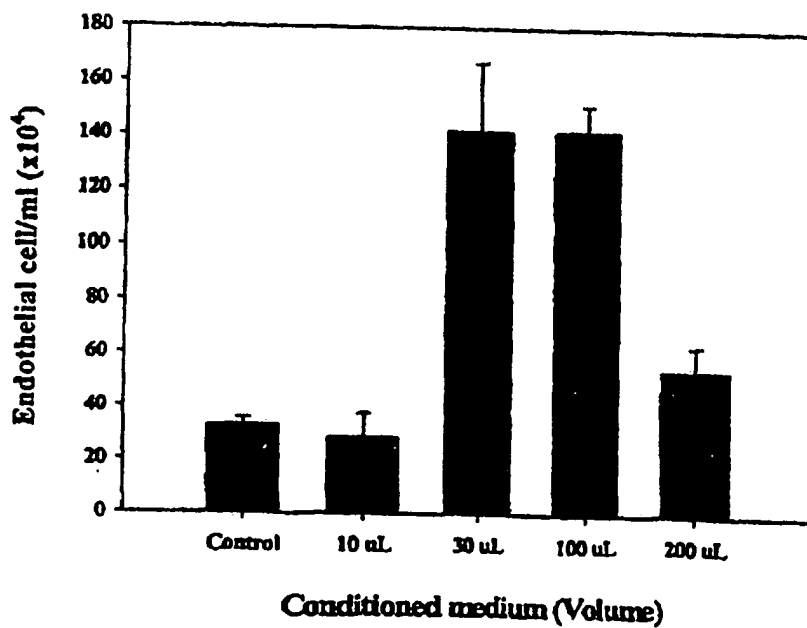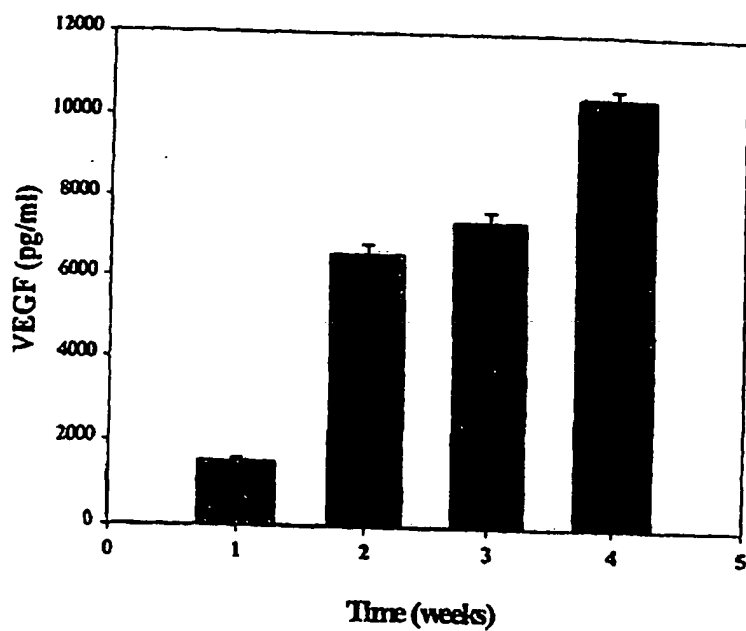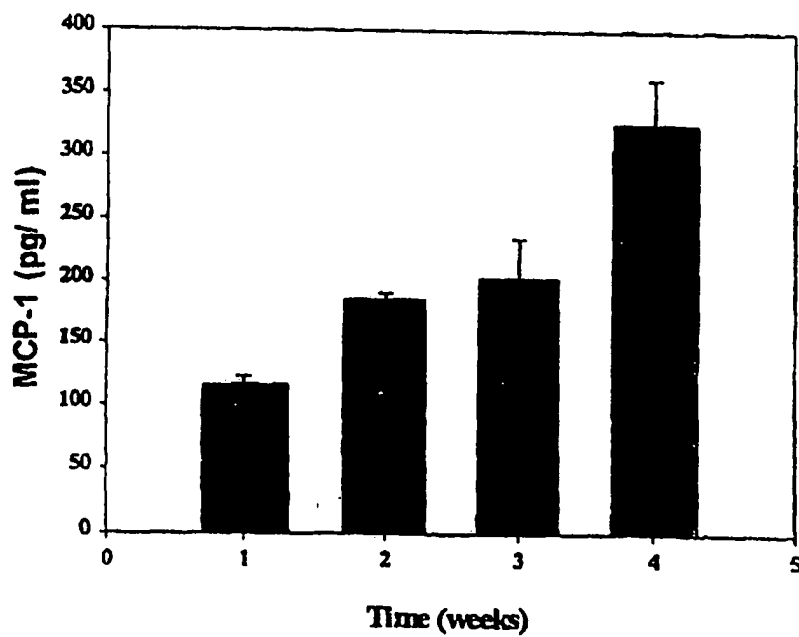
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Fig. 1

Fig. 2

Fig. 3

Fig. 4

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INTRAMYOCARDIAL INJECTION OF AUTOLOGOUS BONE MARROW

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a national stage application of international application PCT/US00/08353, filed 30 Mar. 2000, which claims benefit to U.S. Provisional Application Nos. 60/126,800, filed 30 Mar. 1999, and 60/138,379, filed 9 Jun. 1999.

FIELD OF THE INVENTION

This application is directed to a method of injecting autologous bone marrow. More specifically, this invention is directed to intramyocardial injection of autologous bone marrow to enhance collateral blood vessel formation and tissue perfusion.

BACKGROUND OF THE INVENTION

The use of recombinant genes or growth-factors to enhance myocardial collateral blood vessel function may represent a new approach to the treatment of cardiovascular disease. Kornowski, R., et al., "Delivery strategies for therapeutic myocardial angiogenesis", Circulation 2000; 101:454–458. Proof of concept has been demonstrated in animal models of myocardial ischemia, and clinical trials are underway. Unger, E. F., et al., "Basic fibroblast growth factor enhances myocardial collateral flow in a canine model", Am J Physiol 1994; 266:H1588–1595; Banai, S. et al., "Angiogenic-induced enhancement of collateral blood flow to ischemic myocardium by vascular endothelial growth factor in dogs", Circulation 1994; 83–2189; Lazarous, D. F., et al., "Effect of chronic systemic administration of basic fibroblast growth factor on collateral development in the canine heart", Circulation 1995; 91:145–153; Lazarous, D. F., et al., "Comparative effects of basic development and the arterial response to injury", Circulation 1996; 94:1074–1082; Giordano, F. J., et al., "Intracoronary gene transfer of fibroblast growth factor-S increases blood flow and contractile function in an ischemic region of the heart", Nature Med 1996; 2:534–9. Most strategies for trans-catheter delivery of angiogenic factors have employed an intracoronary route which may have limitations due to imprecise localization of genes or proteins and systemic delivery to non-cardiac tissue. Thus, it would be desirable to have the capacity for direct delivery of angiogenic factors or genes to precisely defined regions of the myocardium rather than to the entire heart, and to minimize the potential for systemic exposure. Guzman, R. J., et al., "Efficient gene transfer into myocardium by direct injection of adenovirus vectors", Circ Res 1993; 73:1202–7; Mack, C. A., et al., "Biologic bypass with the use of adenovirus-mediated gene transfer of the complementary deoxyribonucleic acid for VEGF-121, improves myocardial perfusion and function in the ischemic porcine heart", J Thorac Cardiovasc Surg 1998;115:168–77.

The effect of direct intra-operative intramyocardial injection of angiogenic factors on collateral function has been studied in animal models of myocardial ischemia. Open chest, transepicardial administration of an adenoviral vector containing a transgene encoding an angiogenic peptide resulted in enhanced collateral function. (Mack et al., supra.) Angiogenesis was also reported to occur with direct intramyocardial injection of an angiogenic peptide or a

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plasmid vector during open heart surgery in patients. Schumacher, B., et al., "Induction of neoangiogenesis in ischemic myocardium by human growth factors. First clinical results of a new treatment of coronary heart disease", Circulation 1998; 97:645–650; Losordo, D. W., et al., "Gene therapy for myocardial angiogenesis: initial clinical results with direct myocardial injection of phVEGF165 as sole therapy for myocardial ischemia", Circulation 1998; 98:2800.

Despite the promising hope for therapeutic angiogenesis as a new modality to treat patients with coronary artery disease, there is still a huge gap regarding what specific strategy will optimally promote a clinically relevant therapeutic angiogenic response. Moreover, it is unclear which one (or more) out of multiple angiogenic growth factors may be associated with a beneficial angiogenic response. In addition, the use of different tissue delivery platforms, e.g., proteins, adenovirus, or "naked" DNA, to promote the optimal angiogenic response has remained an open issue.

OBJECTS OF THE INVENTION

It is an object of this invention to provide a novel therapeutic modality wherein autologous bone marrow is injected to promote angiogenesis in the injected tissue.

It is also an object of this invention to provide a novel method of intramyocardial injection to enhance collateral blood vessel formation and tissue perfusion.

These and other objects of the invention will become more apparent in the discussion below.

SUMMARY OF THE INVENTION

Most currently tested therapeutic approaches have focused on a single angiogenic growth factor (e.g., VEGF, FGF, angiopoietin-1) delivered to the ischemic tissue. This can be accomplished either by delivery of the end-product (e.g., protein) or by gene transfer, using diverse vectors. However, it is believed that complex interactions among several growth factor systems are probably necessary for the initiation and maintenance of new blood vessel formation. More specifically, it is believed important to induce a specific localized angiogenic milieu with various angiogenic cytokines interacting in concert and in a time-appropriate manner to initiate and maintain the formation and function of new blood vessels.

The bone marrow (BM) is a natural source of a broad spectrum of cytokines and cells that are involved in the control of angiogenic processes. It is therefore believed that the intramyocardial injection of autologous (A) BM, by taking advantage of the natural ability of these cells to secrete many angiogenic factors in a time-appropriate manner, provides an optimal intervention for achieving therapeutic collateral development in ischemic myocardium.

According to the invention autologous bone marrow is injected, either as a "stand alone" therapeutic agent or combined with any pharmacologic drug, protein or gene or any other compound or intervention that may enhance bone marrow production of angiogenic growth factors and/or promote endothelial cell proliferation, migration, and blood vessel tube formation. The "combined" agent(s) can be administered directly into the patient or target tissue, or incubated ex-vivo with bone marrow prior to injection of bone marrow into the patient. Non-limiting examples of these "combined" agents are Granulocyte-Monocyte Colony Stimulatory Factor (GM-CSF), Monocyte Chemoattractant Protein 1 (MCP-1), and Hypoxia Inducible Factor-1 (HIF-1). An example of an intervention that may enhance bone

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marrow production of angiogenic factors is ex-vivo exposure of bone marrow cells to hypoxia. The autologous bone marrow, alone or with "combined" agents, can be delivered to the patient directly via either trans-endocardial or trans-epicardial approaches into either ischemic and/or non-ischemic myocardium, or directly into any other ischemic organ (including a peripheral limb) to enhance and/or promote the development of collateral blood vessel formation and therefore collateral flow to ischemic myocardium or ischemic limbs. This approach can also be used to promote the development of newly implanted dedifferentiated and/or differentiated myocardial cells by the process of cardiac myogenesis.

The invention comprises various autologous bone marrow transplantation strategies to enhance angiogenesis and/or myogenesis and thereby accelerate the development of new blood vessels into ischemic myocardium or lower extremities. Another aspect of the invention concerns the strategy of "optimization of angiogenic gene expression." This strategy employs co-administration of HIF-1 with the autologous bone marrow. HIF-1 is a transcription factor known to be induced and activated by hypoxia, and known to induce expression of multiple genes involved in the response to hypoxia. A similar approach involves the exposure of autologous bone marrow to endothelial PAS domain protein 1 (EPAS1). EPAS1 shares high structural and functional homology with HIF-1. The strategy also involves the ex-vivo exposure of the bone marrow to hypoxia to increase the production of vascular endothelial growth factor (VEGF) expression or other cytokines with proven angiogene activity (such as MCP-1) prior to its direct injection into the heart or any peripheral ischemic tissue. This invention thus includes the direct intramyocardial (trans-epicardial or trans-endocardial) or peripheral intramuscular injection of autologous bone marrow; stimulated autologous bone marrow, for example, stimulated by HIF-1, EPAS1, MCP-1, GM-CSF, or transient exposure to hypoxia or other forms of energy, such as ultrasound, RF, electromagnetic or laser energy; or autologous bone marrow product derived from conditioned medium (acellular component/s of cultured bone marrow). The stimulation of the bone marrow could be by the direct exposure of the bone marrow to the factors in the form of proteins, or the bone marrow cells can be transfected with vectors carrying the relevant genes. For example, bone marrow can be transfected with a plasmid vector, or with an adenoviral vector, carrying the HIF-1 or EPAS1 transgenes.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graph of the proliferation of PAEC's vs. the quantities of conditioned medium;

FIG. 2 is a graph of the proliferation of endothelial cells vs. the quantities of conditioned medium;

FIG. 3 is a graph of the concentration of VEGF in conditioned medium over a four-week period of time; and

FIG. 4 is a graph of the concentration of MCP-1 in conditioned medium over a four-week period of time.

DETAILED DESCRIPTION OF THE INVENTION

Bone marrow is a natural source of a broad spectrum of cytokines that are involved in the control of angiogenic and inflammatory processes. The cytokines expressed comprise mediators known to be involved in the maintenance of early and late hematopoiesis (IL-1 alpha and IL-1 beta, IL-6, IL-7,

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IL-8, IL-11 and IL-13; colony-stimulating factors, thrombopoietin, erythropoietin, stem cell factor, flt 3-ligand, hepatocyte cell growth factor, tumor necrosis factor alpha, leukemia inhibitory factor, transforming growth factors beta 1 and beta 3; and macrophage inflammatory protein 1 alpha), angiogenic factors (fibroblast growth factors 1 and 2, vascular endothelial growth factor) and mediators whose usual target (and source) is the connective tissue-forming cells (platelet-derived growth factor A, epidermal growth factor, transforming growth factors alpha and beta 2, oncostatin M and insulin-like growth factor-1), or neuronal cells (nerve growth factor). Sensebe, L., et al., *Stem Cells* 1997; 15:133–43. Moreover, it has been shown that VEGF polypeptides are present in platelets and megacaryocytes, and are released from activated platelets together with the release of beta-thromboglobulin. Wartiovaara, U., et al., *Thromb Haemost* 1998; 80:171–5; Mohle, R., *Proc Natl Acad Sci USA* 1997; 94:663–8.

There are also indicators to support the concept that angiogenesis is needed to support bone marrow function and development of hematopoietic cells, including stem cells and progenitor cells, that may enter the circulation and target to sites of wound healing and/or ischemia, ultimately contributing to new blood vessel formation. Monoclonal antibodies that specifically recognize undifferentiated mesenchymal progenitor cells isolated from adult human bone marrow have been shown to recognize cell surface markers of developing microvasculature, and evidence suggests such cells may play a role in embryonal angiogenesis. Fleming, J. E., Jr., *Dev Dyn* 1998; 212:119–32.

Bone marrow angiogensis may become exaggerated in pathologic states where the bone marrow is being activated by malignant cells (such as in multiple myeloma) where bone marrow angiogenesis has been shown to increase simultaneously with progression of human multiple myeloma cells. Ribatti, D., et al., *Br J Cancer* 1999; 79:451–5. Moreover, vascular endothelial growth factor (VEGF) has been shown to play a role in the growth of hematopoietic neoplasms such as multiple myeloma, through either a paracrine or an autocrine mechanism. Bellamy, W. T., *Cancer Res* 1999; 59:728–33; Fiedler, W., *Blood* 1997; 89:1870–5). It is believed that autologous marrow, with its unique native humoral and cellular properties, is a potential source of various angiogenic compounds. This natural source of "mixed" angiogenic cytokines can surprisingly be utilized as a mixture of potent interactive growth factors to produce therapeutic angiogenesis and/or myogenesis; use of the cells per se could provide a more sustained source of these natural angiogenic agents.

One of the factors that most likely participates in initiating angiogenesis in response to ischemia is HIF-1, a potent transcription factor that binds to and stimulates the promoter of several genes involved in responses to hypoxia. Induction and activation of HIF-1 is tightly controlled by tissue pO2; HIF-1 expression increases exponentially as pO2 decreases, thereby providing a positive feedback loop by which a decrease in pO2 causes an increase in the expression of gene products that serve as an adaptive response to a low oxygen environment. Activation of HIF-1 leads, for example, to the induction of erythropoietin, genes involved in glycolysis, and to the expression of VEGF. It probably also modulates the expression of many other genes that participate in the adaptive response to low pO2 levels. The mechanism by which HIF-1 regulates levels of proteins involved in the response to hypoxia is through transcriptional regulation of genes responding to low pO2. Thus, such genes have short DNA sequences within the promoter or enhancer regions

that contain HIF-1 binding sites, designated as hypoxia responsive elements (HRE). HIF-1 is a heterodimer with a basic helix-loop-helix motif, consisting of the subunits HIF-1α and HIF-1β. Its levels are regulated by pO2 both transcriptionally and posttranscriptionally—HIF-1 induction is increased by hypoxia, and its half-life is markedly reduced as pO2 levels increase.

It is relevant that while expression of HIF-1 (as determined in HeLa cells) is exponentially and inversely related to pO2, the inflection point of the curve occurs at an oxygen saturation of 5%, with maximal activity at 0.5% and ½ maximal activity at 1.5–2.0%. These are relatively low levels of hypoxia, and it is not clear whether such levels occur in the presence of mild levels of myocardial or lower limb ischemia—i.e., levels present in the absence of tissue necrosis (myocardial infarction, and leg ulcerations, respectively). Thus, bone marrow cells could have the capacity to secrete angiogenic factors and thereby enhance collateral development. However, it is possible that such activity may not become manifest in the specific tissue environments treated unless some additional stimulus is present. It is, therefore, a preferred aspect of the invention to co-administer, if necessary, bone marrow implant with HIF-1. It is anticipated that HIF-1 will provide optimal expression of many of the hypoxia-inducible angiogenic genes present in the bone marrow implant. The HIF-1 can be injected either as the protein, or as the gene. If as the latter, it can be injected either in a plasmid or viral vector, or any other manner that leads to functionally relevant protein levels. For example, bone marrow can be transfected, ex vivo, with a plasmid vector, or with an adenoviral vector, carrying the HIF-1 or EPSA1 transgenes. It is emphasized, however, that HIF-1 is used in this section as an example of an intervention that could enhance production of angiogenic substances by bone marrow. This invention also covers use of other agents, which by enhancing HIF-1 activity (i.e., prolonging its half-life), or by producing effects analgous to HIF-1, stimulate the bone marrow to increase expression of angiogenic factors. A similar approach involves the exposure of autologous bone marrow to endothelial PAS domain protein 1 (EPAS1). EPAS1 shares high structural and functional homology with HIF-1.

Because VEGF promoter activity is enhanced by HIF-1, this invention also includes the ex-vivo exposure of bone marrow cells in culture to hypoxia or other forms of energy, such as, for example, ultrasound, RF, or electromagnetic energy. This intervention increases VEGF and other gene expression. By this effect it may augment the capacity of bone marrow to stimulate angiogenesis.

Another aspect of the invention involves the ex-vivo stimulation of aspirated autologous bone marrow by HIF-1 (or products that augment the effects of HIF-1 or produce similar effects to HIF-1 on bone marrow) or direct exposure of bone marrow to hypoxic environment followed by the delivery of activated bone marrow cells to the ischemic myocardium or peripheral organ (e.g., ischemic limb) to enhance collateral-dependent perfusion in cardiac and/or peripheral ischemic tissue. The stimulation of the bone marrow could be by the direct exposure of the bone marrow to the factors in the form of proteins, or the bone marrow cells can be transfected with vectors carrying the relevant

genes. For example, bone marrow can be transfected with a plasmid vector, or with an adenoviral vector, carrying the HIF-1 or EPAS1 transgenes.

Current data indicate the importance of monocyte-derived cytokines for enhancing collateral function. Monocytes are activated during collateral growth in vivo, and monocyte chemotactic protein-1 (MCP-1) is upregulated by shear stress in vitro. It has been shown that monocytes adhere to the vascular wall during collateral vessel growth (arteriogenesis) and capillary sprouting (angiogenesis). MCP-1 was also shown to enhance collateral growth after femoral artery occlusion in the rabbit chronic hindlimb ischemia model (Ito et al., *Circ Res* 1997; 80:829–3). Activation of monocytes seems to play an important role in collateral growth as well as in capillary sprouting. Increased monocyte recruitment by LPS is associated with increased capillary density as well as enhanced collateral and peripheral conductance at 7 days after experimental arterial occlusion (Arras M. et al., *J Clin Invest* 1998;101:40–50.)

A further aspect of the invention involves the ex-vivo stimulation of aspirated autologous bone marrow by MCP-1, followed by the direct delivery of activated bone marrow cells to the ischemic myocardium or peripheral organ (e.g., ischemic limb) to enhance collateral-dependent perfusion and muscular function in cardiac and/or peripheral ischemic tissue. The stimulation of the bone marrow could be by the direct exposure of the bone marrow to MCP-1 in the form of the protein, or the bone marrow cells can be transfected with a vector carrying the MCP-1 gene. For example, bone marrow can be transfected with a plasmid vector, or with an adenoviral vector, carrying the MCP-1 transgene.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) and Granulocyte-Colony Stimulatory Factor (G-CSF) are stimulatory cytokines for monocyte maturation and are multipotent hematopoietic growth factors, which are utilized in clinical practice for various hematological pathologies such as depressed white blood cell count (i.e., leukopenia or granulocytopenia or monocytopenia) which occurs usually in response to immunosuppressive or chemotherapy treatment in cancer patients. GM-CSF has also been described as a multilineage growth factor that induces in vitro colony formation from erythroid burst-forming units, eosinophil colony-forming units (CSF), and multipotential (CSF), as well as from granulocyte-macrophage CSF and granulocyte CFU. (Bot F. J., *Exp Hematol* 1989, 17:292–5). Ex-vivo exposure to GM-CSF has been shown to induce rapid proliferation of CD-34+ progenitor cells (Egeland T. et al., *Blood* 1991; 78:3192–9.) These cells have the potential to differentiate into vascular endothelial cells and may naturally be involved in postnatal angiogenesis. In addition, GM-CSF carries multiple stimulatory effects on macrophage/monocyte proliferation, differentiation, motility and survival (reduced apoptotic rate). Consistent with the combined known effects on bone marrow derived endothelial progenitor cells and monocytes, it is another aspect of the invention to use GM-CSF as an adjunctive treatment to autologous bone marrow injections aimed to induce new blood vessel formation and differentiation in ischemic cardiovascular organs. Moreover, GM-CSF may further enhance therapeutic myocardial angiogenesis caused by bone marrow, by augmenting the effect of bone marrow, or by further stimulating, administered either in vivo or in vitro, bone marrow that is also being stimulated by agents such as HIF-1, EPAS1, hypoxia, or MCP-1.

In the examples below, certain testing regarding aspects of the invention is set forth. These examples are non-limitative.

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EXAMPLES

Example 1

Effect of Bone Marrow Cultured Media on Endothelial Cell Proliferation

Studies were conducted to determine whether aspirated pig autologous bone marrow cells obtained secreted VEGF, a potent angiogenic factor, and MCP-1, which recently has been identified as an important angiogenic co-factor. Bone marrow was cultured in vitro for four weeks. The conditioned medium was added to cultured pig aortic endothelial cells (PAECs), and after four days proliferation was assessed. VEGF and MCP-1 levels in the conditioned medium were assayed using ELISA. During the four weeks in culture, BM cells secreted VEGF and MCP-1, such that their concentrations increased in a time-related manner. The resulting conditioned medium enhanced, in a dose-related manner, the proliferation of PAECs. The results indicate that BM cells are capable of secreting potent angiogenic cytokines such as VEGF and MCP-1 and of inducing proliferation of vascular endothelial cells.

Pig Bone Marrow Culture

Bone marrow (BM) cells were harvested under sterile conditions from pigs with chronic myocardial ischemia in preservative free heparin (20 units/ml BM cells) and filtered sequentially using 300µ and 200µ stainless steel mesh filters. BM cells were then isolated by Ficoll-Hypaque gradient centrifugation and cultured in long-term culture medium (LTCM) (Stem Cell Tech, Vancouver, British Columbia, Canada) at 330° C. with 5% CO_2 in T-25 culture flask. The seeding density of the BMCs in each culture was 7×106/ml. Weekly, one half of the medium was removed and replaced with fresh LTCM. The removed medium was filtered (0.2µ filter) and stored at −200° C. for subsequent Enzyme-linked Immunosorbent Assay (ELISA) and cell proliferation assays.

Isolation and Culture of Pig Aortic Endothelial Cells

Fresh pig aortic endothelial cells (PAECs) were isolated using conventional methods. Endothelial cell growth medium (EGM-2 medium, Clonetics, San Diego, Calif.), containing 2% FBS, hydrocortisone, human FGF, VEGF, human EGF, IGF, heparin and antibiotics, at 37° C. with 5% carbon dioxide. When the cells became confluent at about 7 days, they were split by 2.5% trypsin and cultured thereafter in medium 199 with 10% FBS. Their identity was confirmed by typical endothelial cell morphology and by immunohistochemistry staining for factor VIII. Passage 3–10 were used for the proliferation study.

Effects of Conditioned Medium on Aortic Endothelial Cells

Cell proliferation assay: PAECs (Passage 3–10) were removed from culture flasks by trypsinization. The detached cells were transferred to 96-well culture plates and plated at a seeding density of 5,000 cells/well. Cells were cultured for 2–3 days before being used in proliferation and DNA synthesis experiments. The conditioned medium of BM cells cultures were collected at 4 weeks; medium from 7 culture flasks were pooled and used in the bioassay. Aliquotes (10 µL, 30 µL, 100 µL or 200 µL) of pooled conditioned medium, or LTCM (200 µL, as control), were added to confluent PAECs in 96-well plates in triplicate. Four days following culture with conditioned medium or control medium, the PAECs were trypsinized and counted using a cell counter (Coulter Counter Beckman Corporation, Miami Fla.).

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Effects of Conditioned Medium on PAEC DNA Synthesis

Aliquotes (10 µL, 30 µL, 100 µL or 200 µL) of conditioned medium from pooled samples or control medium (LTCM, 200 µL) were added to PAECs in 96-well plate (same seeding density as above) in triplicate. After 2 days, 1 µCi tritiated thymidine was added to each well. Forty-eight hours later, DNA in PAECs was harvested using a cell harvester (Mach III M Tomtec, Hamden, Conn.) and radio-activity was counted by liquid scintillation counter (Multi-detector Liquid Scintillation Luminescence Counter EG&G Wallac, Turku, Finland).

Determination of VEGF and MCP-1 in Conditioned Medium by ELISA VEGF

The concentration of VEGF in conditioned medium was measured using a sandwich ELISA kit (Chemicon International Inc., Temecula, Calif.). Briefly, a plate pre-coated with anti-human VEGF antibody was used to bind VEGF in the conditioned medium or to a known concentration of recombinant VEGF. The complex was detected by the biotinylated anti-VEGF antibody, which binds to the captured VEGF. The biotinylated VEGF antibody in turn was detected by streptavidin-alkaline phosphatase and color generating solution. The anti-human VEGF antibody cross-reacts with porcine VEGF.

Determination of MCP-1 in Conditioned Medium by ELISA

The concentration of MCP-1 in conditioned medium was assayed by sandwich enzyme immunoassay kit (R & D Systems, Minneapolis, Minn.): a plate pre-coated with anti human MCP-1 antibody was used to bind MCP-1 in the conditioned medium or to a known concentration of recombinant protein. The complex was detected by the biotinylated anti-MCP-1 antibody, which binds to the captured MCP-1. The biotinylated MCP-1 antibody in turn was detected by streptavidin-alkaline phosphatase and color generating solution. The anti-human MCP-1 antibody cross-reacts with porcine MCP-1.

Results

The BM conditioned medium collected at four weeks increased, in a dose-related manner, the proliferation of PAECs (FIG. 1). This was demonstrated by counting the number of cells directly and by measuring tritiated thymidine uptake (p<0.001 for both measurements). The dose-related response demonstrated a descending limb; proliferation decreased with 200 µL conditioned medium compared to 30 µL and 100 µL (P 0.003 for both comparisons). Similar dose-related results were observed in the tritiated thymidine uptake studies (P=0.03 for 30 µL and 100 µL compared to 200 µL, respectively).

A limited number (5±4%) of freshly aspirated BM cells stained positive for factor VIII. The results are set forth in FIG. 2. This contrasted to 57±14% of the adherent layer of BM cells cultured for 4 weeks, of which 60±23% were endothelial-like cells and 40±28% appeared to be mega-karyocytes.

Over a 4-week period, the concentrations of VEGF and MCP-1 in the BM conditioned medium increased gradually to 10 and 3 times the 1st week level, respectively (P<0.001 for both comparisons) (FIG. 3). In comparison, VEGF and MCP-1 levels in a control culture medium, not exposed to BM, were 0 and 11±2 pg/ml, respectively, as shown in FIG. 4.

Example 2

Effects of Hypoxia on VEGF Secretion by Cultured Pig Bone Marrow Cells

It was demonstrated that hypoxia markedly increases the expression of VEGF by cultured bone marrow endothelial cells, results indicating that ex-vivo exposure to hypoxia, by increasing expression of hypoxia-inducible angiogenic factors, can further increase the collateral enhancing effect of bone marrow cells and its conditioned media to be injected in ischemic muscular tissue. Pig bone marrow was harvested and filtered sequentially using 300μ and 200μ stainless steel mesh filters. BMCs were then isolated by Ficoll-Hypaque gradient centrifugation and cultured at 33° C. with 5% CO_2 in T-75 culture flasks. When cells became confluent at about 7 days, they were split 1:3 by trypsinization. After 4 wks of culture, the BMCs were either exposed to hypoxic conditions (placed in a chamber containing 1% oxygen) for 24 to 120 hrs, or maintained under normal conditions. The resulting conditioned medium was collected and VEGF, MCP-1 were analyzed by ELISA.

Exposure to hypoxia markedly increased VEGF secretion: At 24 hrs VEGF concentration increased from 106±13 pg/ml under normoxic, to 1,600±196 pg/ml under hypoxic conditions (p=0.0002); after 120 hrs it increased from 4,163±62 to 6,028±167 pg/ml (p<0.0001). A separate study was performed on freshly isolated BMCs, and the same trend was found. Hypoxia also slowed the rate of proliferation of BMCs. MCP-1 expression was not increased by hypoxia, a not unexpected finding as its promoter is not known to have HIF binding sites.

Example 3

Effect of Bone Marrow Cultured Media on Endothelial Cell Tube Formation

It was demonstrated, using pig endothelial cells and vascular smooth muscle cells co-culture technique, that the conditioned medium of bone marrow cells induced the formation of structural vascular tubes in vitro. No such effect on vascular tube formation was observed without exposure to bone marrow conditioned medium. The results suggest that bone marrow cells and their secreted factors exert pro-angiogenic effects.

Example 4

The effect of Transendocardial Delivery of Autologous Bone Marrow on Collateral Perfusion and Regional Function in Chronic Myocardial Ischemia Model

Chronic myocardial ischemia was created in 14 pigs by the implantation of ameroid constrictors around the left circumflex coronary artery. Four weeks after implantation, 7 animals underwent transendocardial injections of freshly aspirated ABM into the ischemic zone using a transendocardial injection catheter (2.4 ml per animal injected at 12 sites) and 7 control animals were injected with heparinized saline. At baseline and 4 weeks later, animals underwent rest and pacing echocardiogram to assess regional contractility (% myocardial thickening), and microsphere study to assess collateral-dependent perfusion at rest and during adenosine infusion. Four weeks after injection of ABM collateral flow (expressed as the ratio of ischemic/normal zone×100)

improved in ABM-treated pigs but not in controls (ABM: 95±13 vs 81±11 at rest, P=0.017; 85±19 vs 72±10 during adenosine, P=0.046; Controls: 86±14 vs 86±14 at rest, P=NS; 73±17 vs 72±14 during adenosine, P=0.63). Similarly, contractility increased in ABM-treated pigs but not in controls (ABM: 83±21 vs 60±32 at rest, P=0.04; 91±44 vs 35±43 during pacing, P=0.056, Controls: 69±48 vs 64±46 at rest, P=0.74; 65±56 vs 37±56 during pacing, P=0.23).

The results indicate that catheter-based transendocardial injection of ABM can augment collateral perfusion and myocardial function in ischemic myocardium, findings suggesting that this approach may constitute a novel therapeutic strategy for achieving optimal therapeutic angiogenesis.

Fourteen specific-pathogen-free domestic pigs weighing approximately 70 kg were anesthetized, intubated, and received supplemental O_2 at 2 L/min as well as 1-2% isoflurene inhalation throughout the procedure. Arterial access was obtained via right femoral artery isolation and insertion of an 8 French sheath. The left circumflex artery was isolated through a left lateral thoracotomy and a metal encased ameroid constrictor was implanted at the very proximal part of the artery. Four weeks after the ameroid constrictor implantation all pigs underwent (1) a selective left and right coronary angiography for verification of ameroid occlusion and assessment of collateral flow; (2) transthoracic echocardiography studies; and (3) regional myocardial blood flow assessment.

Bone Marrow Aspiration and Preparation and Intramyocardial Injection

Immediately after completion of the baseline assessment, all animals underwent BM aspiration from the left femoral shaft using standard techniques. BM was aspirated from 2 sites (3 ml per site) using preservative free heparinized glass syringes (20 unit heparin/1 ml fresh BM). The aspirated bone marrow was immediately macro-filtered using 300% and 200μ stainless steel filters, sequentially. Then, the bone marrow was injected using a trans-endocardial injection catheter into the myocardium in 12 sites (0.2 ml per injection site for total of 2.4. ml) directed to the ischemic myocardial territory and its borderline region.

Echocardiography Study

Transthoracic echocardiography images of short and long axis views at the mid-papillary muscle level were recorded in animals at baseline and during pacing, at baseline and during follow-up evaluation at four weeks after ABM implantation. Fractional shortening measurements were obtained by measuring the % wall thickening (end-systolic thickness minus end-diastolic thickness/end-diastolic thickness)×100. Those measurements were taken from the ischemic territory (lateral area) and remote territory (anterior-septal area). Subsequently, a temporary pacemaker electrode was inserted via a right femoral venous sheath and positioned in the right atrium. Animals were paced at 180/minute for 2 minutes and echocardiographic images were simultaneously recorded.

Regional Myocardial Blood Flow

Regional myocardial blood flow measurements were performed at rest and during maximal coronary vasodilation by use of multiple fluorescent colored microspheres (Interactive Medical Technologies, West Los Angels, Calif.) and quantified by the reference sample technique (Heymann Mass., et al., *Prog Cardiovasc Dis* 1977;20:55-79). Fluorescent microspheres (0.8 ml, 5×10⁶ microspheres/ml, 15 μm diameter in a saline suspension with 0.01% Tween 80) were injected into the left atrium via a 6F Judkins left 3.5 diagnostic catheter. Maximal coronary vasodilation was induced by infusing adenosine at a constant rate of 140

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µg/kg/min (Fujisawa USA, Deerfield, Ill.) into the left femoral vein over a period of 6 minutes. During the last 2 minutes of the infusion, microsphere injection and blood reference withdrawal were undertaken in identical fashion to the rest study.

Following completion of the perfusion assessment, animals were sacrificed with an overdose of sodium pentobarbital and KCL. Hearts were harvested, flushed with Ringer Lactate, perfusion-fixed for 10–15 minutes, and subsequently immersion-fixed with 10% buffered formaldehyde for 3 days. After fixation was completed, the hearts were cut along the short axis into 7-mm thick slices. The 2 central slices were each divided into 8 similar sized wedges, which were further cut into endocardial and epicardial subsegments. The average of 8 lateral ischemic zone and 8 septal normal zone sub-segments measurements were used for assessment of endocardial and epicardial regional myocardial blood flow. The relative collateral flow was also computed as the ratio of the ischemic zone/non ischemic zone (IZ/NIZ) blood flow.

Histopathology

To assess whether injecting BM aspirate via the use of an injection catheter was associated with mechanical cell damage, standard BM smears were prepared before and after propelling the freshly filtered ABM aspirate through the needle using similar injecting pressure as in the in-vivo study. Morphological assessment was performed by an independent experienced technician who was blinded to the study protocol.

Histopathology assessment was performed on sampled heart tissue. In the pilot study, 7-mm thick short-axis slices were examined under UV light to identify fluorescent-tagged areas. Each identified area was cut into 3 full thickness adjacent blocks (central, right and left) that were immersion-fixed in 10% buffered formaldehyde. Subsequently, each such block was cut into 3 levels, of which 2 were stained with Hematoxylin and Eosin (H&E) and one with PAS. In addition, one fresh fluorescent-labeled tissue block was obtained from the ischemic region of each animal and was embedded in OCT compound (Sakura Finetek USA Inc., Torance, Calif.) and frozen in liquid nitrogen. Frozen sections of these snap-frozen myocardial tissue were air dried and fixed with aceton. Immunoperoxidase stain was performed with the automated Dako immunno Stainer (Dako, Carpenteria, Calif.). The intrinsic peroxidase and non-specific uptake were blocked with 0.3% hydrogen peroxidase and 10% ovo-albumin. Monoclonal mouse antibody against CD-34 (Becton Dickinson, San Jose, Calif.) was used as the primary antibody. The linking antibody was a biotinylated goat anti-mouse IgG antibody and the tertiary antibody was strepavidin conjugated with horse reddish peroxidase. Diaminobenzidine (DAB) was used as the chromogen and the sections were counterstained with 1% methylgreen. After dehydration and clearing, the slides were mounted and examined with a Nikon Labphot microscop.

In the efficacy study, full-thickness, 1.5 square centimeter sections from the ischemic and non-ischemic regions were processed for paraffin sections. Each of the samples was stained with H&E, Masson's trichrome, and factor VIII related antigen. The immunoperoxidase stained slides were studied for density of endothelial cell population and vascularization. The latter was distinguished from the former by the presence of a lumen. Vascularity was assessed using 5 photomicrographs samples of the factor VIII stained slides taken from the inner half of the ischemic and non-ischemic myocardium. Density of endothelial cells was assessed using digitized images of the same photomicrographs. The

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density of the endothelial population was determined by Sigma-Scan Pro morphometry software using the intensity threshold method. The total endothelial area for each sample as well as for each specimen were obtained along with the relative percent endothelial area (endothelial area/area of the myocardium studied). The total endothelial area was also calculated as the relative percent of the non-infarcted (viable) area of the myocardium studied. The trichrom stained sections were digitized and the area occupied by the blue staining collagen as well as the total area of the section excluding the area occupied by the epicardium (which normally contained collagen) were measured using Sigma-Scan Pro. The infarcted area was then calculated as the area occupied by the blue staining.

Procedural Data

Intra-myocardial injections either with ABM or placebo were not associated with any acute change in mean blood pressure, heart rate or induction of arrhythmia. All hemodynamic parameters were comparable between the two groups. Pair-wise comparison showed similar hemodynamic parameters within each group in the index compared to the follow-up procedure except for higher initial mean arterial blood pressure at follow-up in the control group (P=0.03) with no subsequent differences during pacing or adenosine infusion.

Myocardial Function

Regional myocardial function assessment is shown in Table I below. Pre-intervention relative fractional wall thickening, expressed as ischemic zone to non-ischemic zone (IZ/NIZ) ratiox100, at rest and during pacing, was similar between groups (P=0.86 and 0.96, respectively). At 4 weeks following the intra-myocardial injection of ABM, improved regional wall thickening occurred at rest and during pacing, which was due to an ~50% increase in wall thickening of the collateral-dependent ischemic lateral wall. No significant changes were observed in the control animals, although a trend towards improvement in wall thickening was noted in the ischemic area during pacing at follow-up.

TABLE I

Regional Contractility of the Ischemic Wall			
	Baseline	Follow-up	P
Rest			
ABM (%)	60 ± 32	83 ± 21	0.04
Control (%)	64 ± 46	69 ± 48	0.74
Pacing			
ABM (%)	36 ± 43	91 ± 44	0.056
Control (%)	37 ± 56	65 ± 56	0.23

ABM indicates autologous bone marrow.

Myocardial Perfusion Data

Regional myocardial perfusion assessment is shown in Table II below. There were no differences between the treated and control groups in the pre-intervention relative transmural myocardial perfusion, IZ/NIZ, at rest and during adenosine infusion (P=0.42 and 0.96, respectively). At 4 weeks following ABM injection, relative regional transmural myocardial perfusion at rest and during pacing improved significantly. This was due to an absolute improvement in myocardial perfusion in the ischemic zone both at rest (an increase of 57%, P=0.08) and during adenosine infusion (37%, P=0.09), while no significant changes were noted in absolute flow to the non-ischemic zone either at rest (increase of 35%, P=0.18) or during adenosine infusion (in-

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crease of 25%, P=0.26). The increase in regional myocardial blood flow found in the ischemic zones consisted of both endocardial (73%) and epicardial (62%) regional improvement at rest, with somewhat lesser improvement during adenosine infusion (40% in both zones). At 4 weeks, the control group showed no differences in transmural, endocardial or epicardial perfusion in the ischemic and non-ischemic zones compared to pre-intervention values.

TABLE II

	Regional Myocardial Perfusion		
	Baseline	Follow-up	P
Rest			
ABM (%)	83 ± 12	98 ± 14	0.001
Control (%)	89 ± 9	92 ± 0.1	0.43
Adenosine			
ABM (%)	78 ± 12	89 ± 18	0.025
Control (%)	77 ± 5	78 ± 11	0.75

ABM indicates autologous bone marrow.

Histopathology and Vascularity Assessment

Assessment of BM smears before and after passing the filtrated aspirate through the injecting catheter revealed normal structure, absence of macro-aggregates and no evidence of cell fragments or distorted cell shapes. Histopathology at day 1 following injections revealed acute lesions characterized by fibrin and inflammatory tract with dispersed cellular infiltration. The infiltrate was characterized by mononuclear cells that morphologically could not be differentiated from a BM infiltrate. Cellularity was maximal at 3 and 7 days and declined subsequently over time. At 3 weeks, more fibrosis was seen in the 0.5 ml injection-sites compared to the 0.2 ml. CD-34 immunostatining, designed to identify BM-derived progenitor cells, was performed in sections demonstrating the maximal cellular infiltrate. Overall, it was estimated that 4–6% of the cellular infiltrate showed positive immunoreactivity to CD-34.

The ischemic territory in both groups was characterized by small areas of patchy necrosis occupying overall <10% of the examined ischemic myocardium. The non-ischemic area revealed normal myocardial structure. Changes in the histomorphometric characteristics of the two groups were compared. There were no differences in the total area occupied by any blood vessel as well as the number of blood vessels >50 μm in diameter. However, comparison of the total areas stained positive for factor VIII (endothelial cells with and without lumen) in the ischemic versus the non-ischemic territories revealed differences between the 2 groups. In the ABM group, the total endothelial cell area in the ischemic collateral-dependent zone was 100% higher than that observed in the non-ischemic territory (11.6±5.0 vs. 5.7±2.3% area, P=0.016), whereas there was no significant difference in the control group (12.3±5.5 vs. 8.2±3.1% area, P=0.11). However, other parameters of vascularity, including % area occupied by any blood vessel and number of blood vessels >50 μm were similar in the ischemic and non-ischemic territories in both groups.

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Example 5

The Effect of Autologous Bone Marrow Stimulated in vivo by Pre-Administration of GM-CSF in Animal Model of Myocardial Ischemia

Chronic myocardial ischemia was created in 16 pigs by the implantation of ameroid constrictors around the left circumflex coronary artery. At four weeks minus 3 days after ameroid implantation, 8 animals underwent subcutaneous injection of GM-CSF for 3 consecutive days (dose 10 μg/kg per day) followed (on the fourth day and exactly 4 weeks after ameroid implantation) by transendocardial injections of freshly aspirated ABM into the ischemic zone using a transendocardial injection catheter (2.4 ml per animal injected at 12 sites) and 8 control animals without GM-CSF stimulation were injected with heparinized saline. At baseline and 4 weeks later, animals underwent rest and pacing echocardiogram to assess regional contractility (% myocardial thickening), and microsphere study to assess collateral-dependent perfusion at rest and during adenosine infusion. Four weeks after injection of ABM collateral flow (expressed as the ratio of ischemic/normal zone×100) improved in ABM-treated pigs but not in controls (ABM: 85±11 vs 72±16 at rest, P=0.026; 83±18 vs 64±19 during adenosine, P=0.06; Controls: 93±10 vs 89±9 at rest, P=0.31; 73±17 vs 75±8 during adenosine, P=0.74). Similarly, contractility increased in ABM-treated pigs but not in controls (ABM: 93±33 vs 63±27 at rest, P=0.009; 84±36 vs 51±20 during pacing, P=0.014, Controls: 72±45 vs 66±43 at rest, P=0.65; 70±36 vs 43±55 during pacing, P=0.18).

The results indicate that catheter-based transendocardial injection of ABM pre-stimulated in vivo by GM-CSF administered systemically for 3 days, can augment collateral perfusion and myocardial function in ischemic myocardium, findings suggesting that this approach may constitute a novel therapeutic strategy for achieving optimal therapeutic angiogenesis.

Example 6

Treatment of a Human Patient

Bone marrow (~5 ml) will be aspirated from the iliac crest at approximately 60 minutes prior to initiation of the cardiac procedure using preservative-free heparinized glass syringes (20 unit heparin/1 ml fresh BM). The aspirated bone marrow will be immediately macro-filtered using 300μ and 200μ stainless steel filters, sequentially. An experienced hematologist will perform the procedure under sterile conditions. The bone marrow smear will be evaluated to confirm a normal histomorphology of the bone marrow preparation.

Any of several procedures for delivery of an agent to the myocardium can be used. These include direct transepicardial delivery, as could be achieved by a surgical approach (for example, but not limited to, a transthoracic incision or transthoracic insertion of a needle or other delivery device, or via thoracoscopy), or by any of several percutaneous procedures. Following is one example of percutaneous delivery. It should be emphasized that the following example is not meant to limit the options of delivery to the specific catheter-based platform system described in the example—any catheter-based platform system can be used.

Using standard procedures for percutaneous coronary angioplasty, an introducer sheath of at least 8F is inserted in the right or left femoral artery. Following insertion of the arterial sheath, heparin is administered and supplemented as

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needed to maintain an ACT for 200–250 seconds throughout the LV mapping and ABM transplantation portion of the procedure. ACT will be checked during the procedure at intervals of no longer than 30 minutes, as well as at the end of the procedure to verify conformity with this requirement.

Left ventriculography is performed in standard RAO and/or LAO views to assist with guidance of NOGA-STAR™ and injection catheters, and an LV electromechanical map is obtained using the NOGA-STAR™ catheter. The 8F INJECTION-STAR catheter is placed in a retrograde fashion via the femoral sheath to the aortic valve. After full tip deflection, the rounded distal tip is gently prolapsed across the aortic valve and straightened appropriately once within the LV cavity.

The catheter (incorporating an electromagnetic tip sensor) is oriented to one of the treatment zones (e.g. anterior, lateral, inferior-posterior or other). Utilizing the safety features of the NOGA™ system, needle insertion and injection is allowed only when stability signals will demonstrate an LS value of <3. A single injection of 0.2 cc of freshly aspirated ABM will be delivered via trans-endocardial approach to the confines of up to two treatment zones with no closer than 5 mm between each injection site. The density of injection sites will depend upon the individual subject's LV endomyocardial anatomy and the ability to achieve a stable position on the endocardial surface without catheter displacement or premature ventricular contractions (PVCs).

That freshly aspirated autologous bone marrow transplanted into ischemic myocardium is associated with improved collateral flow without adverse effects may be of clinical importance for several reasons. The methodology reflected above took advantage of the natural capability of the bone marrow to induce a localized angiogenic response in an effective and apparently safe manner. Such an angiogenic strategy would probably be less costly than many others currently being tested. It would also avoid potential toxicity-related issues that are remote but definite possibilities with various gene-based approaches using viral vectors.

The invention is based on the concept that autologous bone marrow may be an optimal source for cellular (an example would be endothelial progenitor cells, but the invention is not limited to such cells as many other cells in the bone marrow may contribute importantly to the angiogenic effect) and secreted, e.g., angiogenic growth factors, elements necessary to promote new blood vessel growth and restore function when transferred to another tissue, such as ischemic heart or peripheral limbs. A patient's own bone marrow can be used as the key therapeutic source to induce therapeutic angiogensis and/or myogensis in ischemic tissues, e.g., heart muscle and/or ischemic limb, with compromised blood perfusion due to arterial obstructions. The patient's own bone marrow is aspirated, i.e., autologous bone marrow donation, processed, and injected directly into ischemia and/or adjacent non-ischemic tissue, e.g., heart muscle and/or ischemic limb, to promote blood vessel growth.

The autologous bone marrow and/or bone marrow products are injected into the heart muscle, e.g., the myocardium, by use of either a catheter-based trans-endocardial injection approach or a surgical (open chest or via thoracoscopy) trans-epicardial thoracotomy approach. Those two delivery strategies can be used to achieve the same therapeutic goal by promoting the incorporation and integration of angiogenic bone marrow elements in the target organ tissue, e.g., heart muscle and/or ischemic limb.

According to the invention, effective amounts of autologous bone marrow are administered for treatment. As would

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be appreciated by experienced practitioners, the amount administered will depend upon many factors, including, but not limited to, the intended treatment, the severity of a condition being treated, the size and extent of an area to be treated, etc. With regard to treatment according to the invention, a representative protocol would be to administer quantities of from about 0.2 to about 0.5 ml of autologous bone marrow in each of from about 12 to about 25 injections, for a total of from about 2.4 to about 6 ml of autologous bone marrow being administered. Each dose administered could preferably comprise from about 1 to about 2 percent by volume of heparin or another blood anticoagulent, such as coumadin. When the autologous bone marrow has been cultured or stimulated and/or is being administered in combination with other pharmaceuticals or the like, the quantity of autologous bone marrow present should be approximately the same in each dose and/or the total of the autologous bone marrow administered should be about the same as described above. It is believed that the total number of cells of autologous bone marrow administered in each treatment should be on the order of from about 10^7 to 5×10^8.

Optimization of angiogenic gene expression requires the co-administration of various angiogenic stimulants with the autologous bone marrow. Thus, according to the invention autologous bone marrow transplantation is injected either as a "stand alone" therapeutic agent or combined with any pharmacologic drug, protein or gene or any other compound or intervention that may enhance bone marrow production of angiogenic growth factors and/or promote endothelial cell proliferation, migration, and blood vessel tube formation. The "combined" agent(s) can be administered directly into the patient or target tissue, or incubated ex-vivo with bone marrow prior to injection of bone marrow into the patient. Examples of these "combined" agents (although not limited to these agents) are Granulocyte-Monocyte Colony Stimulatory Factor (GM-CSF), Monocyte Chemoattractant Protein 1 (MCP 1), EPAS1, or Hypoxia Inducible Factor-1 (HIF-1). The stimulation of the bone marrow could be by the direct exposure of the bone marrow to the factors in the form of proteins, or the bone marrow cells can be transfected with vectors carrying the relevant genes. For example, bone marrow can be transfected with a plasmid vector, or with an adenoviral vector, carrying the HIF-1 or EPAS1 transgenes. An example of an intervention that may enhance bone production of angiogenic factors is ex-vivo exposure of bone marrow cells to hypoxia. This intervention can be used alone with bone marrow, or in combination with any of the factors outlined above. These optimization strategies are designed to increase the production of vascular endothelial growth factor (VEGF) expression and/or other cytokines with angiogene activity prior to the direct injection of the bone marrow into the heart or any peripheral ischemic tissue. In a broad sense, the invention comprises intramyocardial injection of autologous bone marrow with any agent that would become available to cause stimulation of bone marrow and/or ex-vivo or in vivo stimulation of any angiogenic growth factor production by the bone marrow or its stromal microenvironment.

Delivery to patients will vary, dependent upon the clinical situation. For example, patients with refractory coronary artery disease or ischemic peripheral vasculopathy who will be candidates for a bone marrow aspiration procedure followed by autologous bone marrow myocardial or limb transplantation directed into the ischemic tissue or its borderline zone and/or normal tissue that may serve as the source for collateral or cellular supply to the diseased tissue for the purposes of therapeutic angiogensis and/or myogensis. For

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example, patients with refractory coronary artery disease or ischemic peripheral vasculopathy who will be candidates for a bone marrow aspiration procedure followed by autologous bone marrow myocardial or limb transplantation directed into the ischemic tissue or its bordeline zone and/or normal tissue that may serve as the source for collateral or cellular supply to the diseased tissue for the purposes of therapeutic angiogensis and/or myogensis. This procedure will involve the use of a bone marrow aspiration procedure, bone marrow harvesting and processing, followed by the use of the autologous bone marrow or its elements (growth factors and/or cellular elements being isolated from the patient's own bone marrow), with or without any ex-vivo stimulation of its delivery forms, to be injected into the ischemic or non ischemic myocardium and/or peripheral ischemic tissue (such as limb ischemia). The bone marrow will be kept in standard anti-coagulation/anti-aggregation solution (containing sodium citrate and EDTA) and kept in 4° C. in sterile medium until the time of its use.

Upon its use, the bone marrow will be filtered to avoid injecting remaining blood clots or macroaggregates into the target tissue.

The bone marrow, with or without a stimulatory agent in any of its delivery forms, or with or without having been transfected with a vector carrying a transgene that is designed to enhance the angiogenesis effect of the bone marrow, will be injected into the heart muscle, i.e., in therapeutic myocardial angiogenesis or therapeutic myogensis, using either any catheter-based trans-endocardial injection device or via a surgical (open chest) trans-epicardial thoracotomy approach, or any other approach that allows for transepicardial delivery. In the case of treatment of limb ischemia the bone marrow will be transferred by a direct injection of the bone marrow or it elements, with or without ex-vivo or in vivo stimulation in any of its delivery forms, into the muscles of the leg.

The volume of injection per treatment site will probably range between 0.1–5.0 cc per injection site, dependent upon the specific bone marrow product and severity of the ischemic condition and the site of injection. The total number of injections will probably range between 1–50 injection sites per treatment session.

The preceding specific embodiments are illustrative of the practice of the invention. It is to be understood, however, that other expedients known to those skilled in the art or disclosed herein, may be employed without departing from the spirit of the invention or the scope of the appended claims.

We claim:

1. A method of enhancing collateral blood vessel formation in a subject comprising directly administering to sites in heart or limb tissue an effective amount of autologous bone marrow aspirate to induce collateral blood vessel formation in the tissue.

2. The method of claim 1, wherein the autologous bone marrow aspirate is injected.

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3. The method of claim 1, wherein the autologous bone marrow aspirate is injected intramyocardially.

4. The method of claim 2 wherein the wherein the autologous bone marrow aspirate is injected trans-epicardially or trans-endocardially.

5. The method of claim 4, wherein the trans-endocardial approach is via a catheter.

6. The method of claim 1, wherein the autologous bone marrow aspirate has been stimulated while growing in conditioned medium ex vivo, the conditioned medium comprising at least one agent selected from granulocyte-monocyte colony stimulating factor (GM-CSF), endothelial PAS domain 1 (EPAS1) and hypoxia inducible factor 1 (HIF-1).

7. The method of claim 6, wherein the autologous bone marrow aspirate has been stimulated by contact with one or more angiogenesis stimulating cytokines secreted therefrom while growing in conditioned medium ex vivo, the conditioned medium comprising at least one agent selected from granulocyte-monocyte colony stimulating factor (GM-CSF), endothelial PAS domain 1 (EPAS1) and hypoxia inducible factor 1 (HIF-1).

8. The method of claim 1, wherein the autologous bone marrow aspirate further comprises Monocyte Chemoattractant Protein 1 (MCP-1) or Vascular Endothelial Growth Factor (VEGF).

9. The method of claim 6, wherein the autologous bone marrow aspirate has been stimulated ex vivo in culture by transient exposure to hypoxia.

10. The method of claim 1, wherein the autologous bone marrow aspirate is administered in combination with one or more agent selected from a pharmacological drug or protein that enhances bone marrow production of angiogenic growth factors selected to promote endothelial cell proliferation, migration, or blood vessel formation.

11. The method of claim 10, wherein the autologous bone marrow aspirate and the agent or agents are administered together.

12. The method of claim 10, wherein the autologous bone marrow aspirate and the agent or agents are combined ex vivo prior to administration.

13. The method of claim 12, wherein the autologous bone marrow aspirate has been stimulated ex vivo in conditioned medium, the conditioned medium comprising at least one agent selected from granulocyte-monocyte colony stimulating factor (GM-CSF), endothelial PAS domain 1 (EPAS1) and hypoxia inducible factor 1 (HIF-1).

14. The method of claim 1, wherein the autologous bone marrow aspirate is administered to ischemic tissue.

15. The method of claim 12, further comprising culturing the autologous bone marrow aspirate to form conditioned medium containing bone marrow cells and endogenously secreted angiogenic cytokines and injecting the composition into ischemic heart tissue.

* * * * *

EVIDENCE APPENDIX

ITEM NO. 27

Third Supplemental Declaration of Dr. Heuser (originally filed in co-pending application Serial No. 10/179,589) and cited by Appellant as Exhibit B in the Letter filed May 25, 2007

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: James P. Elia)	
)	Group Art Unit: 1646
Serial No.: 10/179,589)	
)	Examiner: Daniel C. Gamett
Filed: June 25, 2002)	
)	
For: METHOD FOR GROWING)	
HUMAN ORGANS AND)	
SUBORGANS)	

THIRD SUPPLEMENTAL DECLARATION
OF RICHARD HEUSER, M.D., F.A.C.C., F.A.C.P.

I Richard Heuser declare as follows:

1.   I have offices at 500 West Thomas Road, Suite 900, Phoenix, Arizona 85013.

2.   My Curriculum Vitae was attached as Exhibit A to my Declaration of November 16, 2004.   Paragraph 3 of my Declaration and my Supplemental Declaration of February 15, 2005 provide additional information regarding my background and experience.

3.   I have read the Examiner's criticism contained in paragraph 11, commencing on page 7 and ending on page 9 of the March 7, 2007 Office Action regarding the conversion of dosages of plasmid cDNA to dosages of cells.   Such paragraph is set forth in Third Supplemental Exhibit A attached hereto.   Specifically, I note the Examiner's criticism bridging pages 7 and 8 regarding the above-mentioned conversion that:

...one of skill in the art would never think to
attempt such an extrapolation. The unsound
scientific basis for the conversion of plasmid DNA
to cellular equivalents would be obvious to anyone
trained in molecular biology.

4. I have read and understood the disclosures of the above-referenced patent
application at page 4, line 1 through page 5, line 14; at page 13, lines 3-10;
at page 22, line 5 through page 24, line 15; and at page 26, line 3 through
page 27, line 3. A copy of such disclosures is attached hereto as Third
Supplemental Declaration Exhibit B.

I have also read and understood additional disclosures of the above-
referenced patent application at page 9, lines 14-16; page 17, line 1
through page 20 line 8; page 21, lines 23 and 24; page 27, lines 1-3; page
28, lines 12-16; page 32, line 20 through page 39, line 19; and page 44,
lines 8-17. A copy of such additional disclosures is attached hereto as
Third Supplemental Declaration Exhibit C.

5. I have read and understood Applicant's conversion for dosages of plasmid
cDNA to equivalent corresponding dosages of cells set forth in attached
Third Supplemental Exhibit D as it relates to Examples 18 and 17 of the
specification, which are contained in Third Supplemental Declaration
Exhibit C.

6. In my opinion, the Examiner's criticism specifically delineated in
Paragraph 3 above is not credible. Contrary to the Examiner's opinion,
studies involving conversion of the average (mean) content of nucleic
acids per cell in human marrow cells have been routinely conducted and
accepted by skilled scientists for over 50 years. Three (3) publications
illustrating the use of such well known conversion are included in the
attached Third Supplemental Declaration Exhibit E. Note that in two of

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the publications, typical conversion results are set forth in tables, thereby eliminating the necessity to perform the actual calculation. Obviously, a sound scientific basis exists in the medical art for such conversions.

Further, those skilled in the art understand that DNA content is substantially consistent from tissues of any given species. Consequently, a skilled medical person relying on sound scientific bases at the time of the present invention would reasonably have understood how to extrapolate plasmid DNA to cells on a weight basis. Applicant's use of 40 pg as an average weight for nucleic acids in a human cell is fairly representative. Thus, I find Applicant's conversion set forth in the attached Third Supplemental Declaration Exhibit D to be consistent with the extrapolations set forth above and commonly used and relied upon by skilled persons in the medical art. Accordingly, the dosages specified in Examples 18 and 17 are sufficient to enable a person skilled in the medical art to convert dosages of plasmid DNA to corresponding dosages of genomic DNA within the context of Applicant's disclosed invention.

7. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date:_____ _____

Richard Heuser

3

THIRD SUPPLEMENTAL SPECIFICATION

EXHIBIT

MARCH 2007
Office Action
Palate... notes 7.9

11.   Applicant admits on page 9 that Examples 17-19 employ nucleic acids, but asserts that

one skilled in the art reading the specification, which teaches that cells, i.e., stem cells (BMC's)

possess equivalent activity to genes (nucleic acids) and other genetic material in forming a new

artery (i.e., promote morphogenesis of an organ—artery), would be able to easily extrapolate the

number on a weight basis of mononuclear cells required to obtain equivalent results. According

to the method for extrapolation provided in the footnote to pages 10-11, 250 µg of plasmid DNA

(an amount described in Examples 17 and 18) divided by 40 pg, (asserted to be is the average

DNA content of a cell; the species of cell is not disclosed) equals 6.25×10^6, and therefore the

Examples 17 and 18 instruct the skilled artisan to use 6.25×10^6 cells. This argument is not

persuasive for several reasons. First, this method of converting plasmid DNA to cell equivalents

is not included in the specification as filed. This is important because one of skill in the art would

never think to attempt such an extrapolation. The unsound scientific basis for the conversion of

µg of plasmid DNA to cellular equivalents would be obvious to anyone trained in molecular

biology. One basic assumption of the recited conversion is that the 40 pg of cellular DNA comprises the same gene dosage a purified plasmid DNA. Every molecule of the postulated plasmid DNA comprises a copy of the VEGF cDNA. In contrast, VEGF coding sequences would comprise but one of 30-40 thousand genes in genomic DNA (at the time of filing, it was widely believed that the human genome comprised 100,000 genes). Therefore, one of skill in the art at the time of filing would not expect plasmid DNA and genomic DNA to be comparable on a per weight basis. Applicant's argument seems to view the living cell as little more than a container for DNA. The expression of the recombinant cDNA would be under control of the limited number of enhancer and promoter elements in the plasmid, as opposed the native control elements with the genome. Therefore, even equivalent gene doses would not be expected to yield equivalent amounts of gene product with a plasmid as opposed to a cell. Applicant's argument seems to view the living cell as little more than a container for DNA. Delivery of the genes to a target as recombinant DNA as opposed to native genes within a living cell are technically different processes; there is no basis for using one to guide the other. For example, with DNA one is concerned with chemical stability, efficiency of uptake, stable retention, and subsequent expression of the injected molecule into target cells, whereas with cells separate issues of formation of effective attachment to ECM and neighboring cells, short- and long-term viability, and responses to environmental cues arise. As evidence, one need look no further than the US Patent classification system. Methods of *in vivo* treatments involving whole live cells as opposed to nucleic acids are separately classified: class 424 subclass 93.1 (cells); class 514, subclass 44 (polynucleotides). These separate classifications indicate a different status in the art such that it is well known that cell therapy and gene therapy are not obvious variants of one

- A2 -

another. Therefore, contrary to Applicants assertion on page 9, the specification does not

describe any dosage of cells to use to promote artery growth.

THIRD SUPPLEMENTAL
DECLARATION

EXHIBIT B

DISCLOSURE

PAGE 4, LINE 1 – PAGE 5, LINE 14

Growth factors can be utilized to induce the growth of "hard tissue" or bone and "soft tissues" like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basis (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor an also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which an often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 13, LINES 3-10

Multifactorial and nonspecific cells (such as stem cells and germinal cells) can provide the necessary *in vivo* and *in vitro* cascade of genetic material once an implanted master control gene's transcription has been activated. Likewise, any host cell, cloned cell, cultured cell, or cell would work. Genetic switches (such as the insect hormone ecdysone) can be used to control genes inserted into humans and animals. These gene switches can also be used in cultured cells or other cells. Gene switches govern whether a gene is on or off making possible precise time of gene activity.

PAGE 22, LINE 5 – PAGE 24, LINE 15

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in connection with any of the implant techniques of the invention, it is understood that a cell nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated

(taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth

factors (or other genetic material) are placed. Once the new muscle and blood vessels have grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

PAGE 26, LINE 3 – PAGE 27, LINE 3

Organs and/or tissues can be formed utilizing the patient's own cells. For example, a skin cell(s) is removed from the intraoral lining of a cheek. The cell is genetically screened to identify DNA damage or other structural and/or functional problems. Any existing prior art genetic screening technique can be utilized. Such methods can utilize lasers, DNA probes, PCR, or any other suitable device,. If the cell is damaged, a healthy undamaged cell is, if possible, identified and selected. If a healthy cell cannot be obtained, the damaged cell can be repaired by excision, alkylation, transition, or any other desired method. A growth factor(s) is added to the cell to facilitate dedifferentiation and then redifferentiation and morphogenesis into an organ or function specific tissue. Any machine known in the art can be used to check the genetic fitness of the organ and its stage of morphogenesis. A cell nutrient culture may or may not be utilized depending on the desired functional outcome (i.e., growth of an artery, of pancreatic Islet cells, of a heart, etc.) or other circumstances. Replantation can occur at any appropriate stage of

morphogenesis. The foregoing can be repeated without the patient's own cells if universal donor cells such as germinal cells are utilized. Germinal cells do not require a dedifferentiation. They simply differentiate into desired tissues or organs when properly stimulated. Similarly, the DNA utilized in the foreign procedure can come from the patient or from any desired source.

During reimplantation one of the patient's own cells is returned to the patient. During implantation, a cell not originally obtained from the patient is inserted on or in the patient.

In the example above, if germinal cells (and in some cases, stem cells) are utilized, a direct differentiation and morphogenesis into an organ can occur *in vivo, ex vivo,* or *in vitro.*

THIRD SUPPLEMENTAL
DECLARATION

EXHIBIT C

ADDITIONAL
DISCLOSURES

EXHIBIT C

DISCLOSURES
APPLICATION SERIAL NO. 10/179,589

PAGE 9, LINES 14-16

Morphogenesis or morphogenetics is the origin and evolution of morphological characters and is the growth and differentiation of cells and tissues during development.

PAGE 17, LINE 1 – PAGE 20, LINE 8

EXAMPLE 10

MSX-1 and MSX-2 are the homeobox genes that control the generation and growth of a tooth. A sample of skin tissue is removed from the patient and the MSX-1 and MSX-2 homeobox gene(s) are removed from skin tissue cells. The genes are stored in an appropriate nutrient culture medium.

BMP-2 and BMP-4 growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 and 102 degrees F.

MSX-1 and MSX-2 transcription factors are obtained which will initiate the expression of the MSX-1 and MSX-2 homeobox genes.

The MSX-1 and MSX-2 transcription factors, BMP-2 and MBP-4 bone morphogenic proteins, and MSX-1 and MSX-2 genes are added to the nutrient culture medium along with the living stem cells.

EXAMPLE 11

Example 10 is repeated except that the transcription factors bind to a receptor complex in the stem cell nucleus.

EXAMPLE 12

Example 10 is repeated except that the MSX-1 and MSX-2 transcription factors are not utilized. The transcription of the MSX-1 and MSX-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 13

Example 10 is repeated except that the stem cells are starved and the transcription of the MSX-1 and MSX-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 14

WT-1 and PAX genes are obtained from a sample of skin tissue removed from the patient. The genes are stored in an appropriate nutrient culture medium. PAX genes produce PAX-2 and other transcription factors.

BMP-7 and other kidney related BMP growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

The WT-1 and PAX genes, and BMP-7 and other kidney BMPS are added to the nutrient culture medium along with the living stem cells.

A primitive kidney germ is produced. The kidney germ is transplanted in the patient's body near a large artery. As the kidney grows, its blood supply will be derived from the artery.

EXAMPLE 15

The Aniridia gene is obtained from a sample of skin tissue removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

The Aniridia transcription factor and growth factor and the Aniridia gene are added to the nutrient culture medium along with the living stem cells.

A primitive eye germ is produced. The eye germ is transplanted in the patient's body near the optic nerve. As the eye grows, its blood supply will be derived from nearby arteries.

EXAMPLE 16

The Aniridia gene is obtained from a sample of skin tissue removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained and added to the nutrient culture medium.

An eye germ develops. A branch of the nearby maxillary artery is translocated to a position adjacent the eye germ to promote the development of the eye germ. The eye germ matures into an eye which receives its blood supply from the maxillary artery.

The term "cell nutrient culture" as used herein can include any or any combination of the following: the extracellular matrix; conventional cell culture nutrients; and/or, a cell nutrient such as a vitamin. As such, the cell nutrient culture can be two-dimensional, three-dimensional, or simply a nutrient, and is useful in promoting the processes of cellular dedifferentiation, redifferentiation, differentiation, growth, and development.

PAGE 21, LINES 23– 24

An organ, as used herein, consists of two or more kinds of tissues joined into one structure that has a certain task.

- C4 -

PAGE 27, LINES 1-3

In the example above, if germinal cells (and in some cases, stem cells) are utilized a direct differentiation and morphogenesis into an organ can occur *in vivo*, *ex vivo*, or *in vitro*.

PAGE 28, LINES 12-16

Avascular necrosis can be corrected with the insertion of a gene(s) and/or growth factor or other genetic material in the body. For example, avascular necrosis is diagnosed near a joint space. VEGF or BMP genes, or VEFG or BMP growth factors produced by VEFG or BMP genes, respectively, or any other desired genetic based material can be inserted to regrow blood vessels and/or bone.

PAGE 32, LINE 20 – PAGE 39, LINE 19

EXAMPLE 17

A 36-year old Caucasian male experiences pain in his left leg. A medical examination reveals a damaged one-inch long section of a large artery in his left leg. The examination also reveals that this damaged section of the artery is nearly completely clogged with plaque and that the wall of the artery is weakened. The weakening in the arterial wall makes attempting to clean out the artery risky and also makes it risky to attempt to insert a stent in the artery.

Recombinant cDNA encoded to combine with a cell ribosome to produce the human growth factor VEGF is assembled into a eukaryotic expression plasmid. The recombinant

cDNA is from cDNA libraries prepared from HL60 leukemia cells and is known to cause the growth of arteries. The plasmid is maintained at a room temperature of 76 degrees F.

The clones are placed in 1.0 milliliters of a normal saline carrier solution at a room temperature of 76 degrees F. to produce a genetic carrier solution. The genetic carrier solution contains about 250 ug of the cDNA clones. A nutrient culture can, if desired, be utilized in conjunction with or in place of the saline carrier. Each clone is identical. If desired, only a single clone can be inserted in the normal saline carrier solution. The saline carrier solution comprises 0.09% by weight sodium chloride in water. A saline carrier solution is selected because it will not harm the DNA clone.

Two sites are selected for injection of the genetic carrier solution. While the selection of sites can vary as desired, the sites are selected at the lower end (the end nearest the left foot of the patient) of the damaged section of the artery so that the new arterial section grown, can, if necessary, be used to take the place of the damaged section of the artery in the event the damaged section is removed.

The first site is on the exterior wall of the artery on one side of the lower end of the damaged section of the artery. A containment system is placed at the first site.

The second site is inside the wall of the artery on the other side of the lower end of the artery.

The genetic carrier solution is heated to a temperature of 98.6 degrees F. 0.25 milliliters of the genetic carrier solution is injected into the containment system at the first site. 0.25 milliliters of the genetic carrier solution is injected at the second site inside the wall of the artery. Care is taken to slowly inject the genetic carrier solution to avoid entry of the solution into the artery such that blood stream will carry away the cDNA in the solution.

After two weeks, an MRI is taken which shows the patient's leg artery. The MRI reveals new growth at the first and second sites.

After four weeks, another MRI is taken which shows the patient's leg artery. The MRI shows that (1) at the first site, a new artery is growing adjacent the patient's original leg artery, and (2) at the second site, a new section of artery is growing integral with the original artery, i.e., at the second site the new section of artery is lengthening the original artery, much like inserting a new section of hose in a garden hose concentric with the longitudinal axis of the garden hose lengthens the garden hose.

After about eight to twelve weeks, another MRI is taken which shows that the new artery growing adjacent the patient's original artery has grown to a length of about one inch and has integrated itself at each of its ends with the original artery such that blood flows through the new section of artery. The MRI also shows that the new artery at the second site has grown to a length of one-half inch.

In any of the examples of the practice of the invention included herein, cell nutrient culture can be included with the gene, the growth factor, the extracellular matrix, or the environmental factors.

In any of the examples of the practice of the invention included herein, the concept of gene redundancy can be applied. For example, the Examples 1 to 14 concerning a tooth list the genes MSX-1 and MSX-2. These genes differ by only two base pairs. Either gene alone may be sufficient. A further example of redundancy occurs in growth factors. Looking at the Examples 10 to14, BMP4 or BMP2 alone may be sufficient. Redundancy can also be utilized in connection with transcription factors, extracellular matrices, environmental factors, cell nutrient culture, physiological nutrient cultures, vectors, promoters, etc.

One embodiment of the invention inserts genetic material (gene, growth factor, ECM, etc.) into the body to induce the formation of an organ. Similar inducing materials *ex vivo* into or onto a living cell in an appropriate physiological nurturing environment will also induce the growth of an organ. The VCSEL laser allows early detection in a living cell of a morphogenic change indicating that organ formation has been initiated. With properly time transplantation, organ growth completes itself.

During the *ex vivo* application of the invention, a gene and/or growth factor is inserted into a cell or a group of cells; an ECM or environmental factor(s) are placed around and in contact with a cell or group of cells; or, genetic material is inserted into a subunit of a cell to induce organ growth. An example of a subunit of a cell is an enucleated cell or a comparable artificially produced environment. In *in vivo* or *ex vivo* embodiments of the invention to induce the growth of an organ, the genes, growth factors, or other genetic material, as well as the environmental factors or cells utilized, can come from any desired source.

EXAMPLE 18

Genetically produced materials are inserted in the body to cause the body to grow, reproduce, and replace *in vivo* a clogged artery in the heart. This is an example of site-specific gene expression. A plasmid expression vector containing an enhancer/promoter is utilized to aid in the transfer of the gene into muscle cells. The enhancer is utilized to drive the specific expression of the transcriptional activator. After the enhancer drives the expression of the transcriptional activator, the transcriptional activator transactivates the muscle/artery genes. Saline is used as a carrier. Cardiac muscle can take up naked DNA injection intramuscularly.

Injecting plasmid DNA into cardiac (or skeletal) muscle results in expression of the transgene in cardiac myocytes for several weeks or longer.

Readily available off-the-shelf (RAOTS) cDNA clones for recombinant human VEFG165, isolated from cDNA libraries prepared from HL60 leukemia cells, are assembled in a RAOTS expression plasmid utilizing 736bp CMV promoter/enhancer to drive VEGF expression. Other RAOTS promoters can be utilized to drive VEGF expression for longer periods of time. Other RAOTS recombinant clones of angiogenic growth factors other than VEGF can be utilized, for example, fibroblast growth factor family, endothelial cell growth factor, etc. Downstream from the VEGF cDNA is an SV40 polyadenylation sequence. These fragments occur in the RAOTS pUC118 vector, which includes an Escherichia coli origin of replication and the Beta lactamase gene for ampicillin resistance.

The RAOTS construct is placed into a RAOTS 3 ml syringe with neutral pH physiologic saline at room temperature (or body temperature of about 73 degrees C). The syringe has a RAOTS 27 gauge needle.

Access to the cardiac muscle is gained by open-heart surgery, endoscopic surgery, direct injection of the needle with incision, or by any other desired means. The cardiac muscle immediately adjacent a clogged artery is slowly injected with the RAOTS construct during a five second time period. Injection is slow to avoid leakage through the external covering of muscle cells. About 0.5 ml to 1.0 ml (milliliter) of fluid is injected containing approximately 500 ug phVEGF165 in saline (N=18). The readily available off-the-shelf cDNA clones cause vascular growth which automatically integrates itself with the cardiac muscle. Anatomic evidence of collateral artery formation is observed by the 30th day following injection to the RAOTS construct. One end of the artery integrates itself in the heart wall to receive blood from the heart.

The other end of the artery branches into increasingly smaller blood vessels to distribute blood into the heart muscle. Once the growth of the new artery is completed, the new artery is left in place in the heart wall. Transplantation of the new artery is not required.

Blood flow through the new artery is calculated in a number of ways. For example, Doppler-derived flow can be determined by electromagnetic flowmeters (using, for example, a Doppler Flowmeter sold by Parks Medical Electronic of Aloha, Oregon) both *in vitro* and *in vivo*. Also, RAOTS angiograms or any other readily available commercial devices can be utilized.

VEGF gene expression can be evaluated by readily available off-the-shelf polymerase chain reaction (PCR) techniques.

If controls are desired, the plasmid pGSVLacZ containing a nuclear targeted Beta-galactosidase sequence coupled to the simian virus 40 early promoter can be used. To evaluate efficiency, a promoter-matched reporter plasmid, pCMV Beta (available from Clontech of Palo Alto, California), which encodes Beta-galactosidase under control of CMV promoter/enhancer, can be utilized. Other RAOTS products can be utilized if desired.

EXAMPLE 19

A patient, a forty-year old African-American female in good health, has been missing tooth number 24 for ten years. The space in her mouth in which her number 24 tooth originally resided is empty. All other teeth except tooth number 24 are present in the patient's mouth. The patient desires a new tooth in the empty "number 24" space in her mouth.

A full thickness mucoperiosteal flap surgery is utilized to expose the bone in the number 24 space. A slight tissue reflection into the number 23 tooth and number 25 tooth areas is carried out to insure adequate working conditions.

A Midwest Quietair handpiece (or other off-the-shelf handpiece) utilizing a #701 XXL bur (Dentsply Midwest of Des Plaines, Illinois) (a #700, #557, #558, etc. bur can be utilized if desired) is used to excavate an implant opening or site in the bone. The implant opening is placed midway between the roots of the number 23 and number 25 teeth. The opening ends at a depth which is about fifteen millimeters and which approximates the depth of the apices of the roots of the number 23 and number 25 teeth. Care is taken not to perforate either the buccal or lingual wall of the bone. In addition, care is taken not to perforate or invade the periodontal ligament space of teeth numbers 23 and 25.

An interrupted drilling technique is utilized to avoid overheating the bone when the #701XXL bur is utilized to form the implant opening. During a drilling sequence, the drill is operated in five-second increments; and the handpiece is permitted to stall. Light pressure and a gentle downward stroke are utilized.

PAGE 44, LINES 8–17

EXAMPLE 35

Example 17 is repeated except that the patient is a 55-year old Caucasian male, and the genetic carrier solution is injected into two sites in the coronary artery of the patient. The first site is on the exterior wall on one side of the artery. The second site is inside the wall of the artery on the other side of the artery. A section of the artery is damaged, is partially blocked, and has a weakened wall. The first and second sites are each below the damaged section of the

artery. Similar results are obtained, i.e., a new section of artery grows integral with the original artery, and a new section of artery grows adjacent the original artery. The new section of artery has integrated itself at either end with the original artery so that blood flows through the new section of artery.

EXHIBIT D

CONVERSION

The conversion for dosages of nucleic acids to corresponding dosages of cells was conducted as follows. Examples 18 and 17 specified dosages of 500 micrograms (ug) and 250 ug, respectively. The weight of nucleic acids of an average cell was considered to equal 40 picograms (pg). The described dosages of 250 and 500 ug when converted to pg by multiplying by 10^6 equals 250×10^6 pg and 500×10^6 pg. Since nucleic acids of an average cell have an average weight of 40 pg, a conversion is made by dividing 250×10^6 and 500×10^6 by 40 to arrive at the equivalent cell dosages, which are 6.25×10^6 and 12.5×10^6, respectively.

THIRD SUPPLEMENTAL
DECLARATION

EXHIBIT

PROVISIONS (?)

Studies on the Average Content of Nucleic Acids in Human Marrow Cells. By J. N. DAVIDSON, I. LESLIE and J. C. WHITE. (*From the Department of Biochemistry, University of Glasgow, and the Department of Pathology, Postgraduate Medical School of London*)

In extension of previously reported analyses of the deoxyribonucleic acid phosphorus (DNAP) and ribonucleic acid phosphorus (RNAP) content of aspirated human bone marrow (Davidson, Leslie & White, 1947, 1948), we now report a modification involving enumeration of the nucleated cell content of the samples analysed. Results are expressed in terms of DNAP and RNAP per cell (Table 1), and are average values for the growing and adult cell populations of the analysed samples. The recent results of Vendrely & Vendrely (1948, 1949) and of Mirsky & Ris (1949) suggest a striking constancy in the DNAP content of normal cell nuclei from the tissues of any given species, and our figures for DNAP are of the same order as those quoted by the Vendrelys for human liver nuclei.

There is no significant difference between the means for the normal and the leukaemic series, either as a whole, or considering only acute leukaemia prior to therapy.

A small series of 6 cases of iron-deficiency anaemia has not shown significant variation of the mean DNAP and RNAP per cell from normal.

Results obtained from cases of pernicious and other megaloblastic anaemias are shown in Tables 2 and 3.

It must be noted clearly that the group under therapy cannot be considered as returned to normal, either as regards blood picture, marrow cytology or adequacy of therapy. The significant fall in RNAP from that in the group prior to therapy parallels the general increase in maturity of the marrow under therapy. Cases fully treated and returned to normal are under investigation.

Table 1

Normal human marrow

Values of Nucleic Acid Phosphorus (NAP) in
$\mu g. \times 10^{-7}$ per cell

	DNAP 18 obs. on 16 individuals	RNAP 20 obs. on 18 individuals	Ratio RNAP/ DNAP
Mean	8·54	6·33	0·75
s.e. of obs.	2·89	3·03	0·326
Observed range	4·0–15·0	2·1–13·5	0·43–1·9

Marrow from cases of leukaemia of various types, before and during therapy

	28 obs. on 15 cases	24 obs. on 12 cases	
Mean	8·75	7·59	0·90
s.e. of obs.	3·05	3·72	0·30
Observed range	3·9–17·4	2·0–17·4	0·3–1·8

Table 2. Cases of pernicious anaemia and other megaloblastic anaemias

NAP in $\mu g. \times 10^{-7}$ per cell

Group as a whole		DNAP 28 obs. on 12 cases	RNAP	Ratio DNAP/RNAP 28 obs. on 13 cases
	Mean	12·6	10·9	0·87
	s.e.	4·56	5·03	0·27
	Observed range	6·6–22·8	2·3–25·1	0·35–1·5
Group prior to therapy		12 obs. on 12 cases	11 obs. on 11 cases	12 obs. on 12 cases
	Mean	12·57	13·38	1·06
	s.e.	4·17	5·19	0·249
	Observed range	8·1–22·8	7·5–25·1	0·69–1·5
Group during the course of therapy		17 obs. on 8 cases	15 obs. on 8 cases	16 obs. on 9 cases
	Mean	12·63	9·09	0·73
	s.e.	4·36	4·21	0·198
	Observed range	6·6–16·8	2·3–17·6	0·35–1·0

Table 3. t test of significance between means

		DNAP	RNAP	Ratio RNAP/DNAP
Megaloblastic series as a whole compared with normal series	P Degrees of freedom	<0·001 44 Highly significant	<0·001 44 Highly significant	0·2–0·1 46 Not significant
Megaloblastic series before therapy compared with normal	P Degrees of freedom	0·01–0·001 28 Highly significant	<0·001 29 Highly significant	0·01–0·001 30 Highly significant
Megaloblastic series during therapy compared with normal	P Degrees of freedom	0·01–0·001 33 Highly significant	0·05–0·02 33 Significant	0·8–0·7 34 Not significant
Megaloblastic series before and during therapy compared	P Degrees of freedom	0·7–0·6 27 Not significant	0·05–0·02 24 Significant	<0·001 26 Highly significant

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Fluoroacetate Poisoning and 'Jamming' of the Tricarboxylic Acid Cycle; Mode of Action of an 'Active' Fluoro Compound Synthesized via this Cycle. By P. BUFFA, W. D. LOTSPEICH, R. A. PETERS and R. W. WAKELIN. (*Department of Biochemistry, University of Oxford*)

So far no isolated enzyme has been inhibited by fluoroacetate. The hypothesis has been advanced by Liébecq & Peters (1949) (see also Martius, 1949) that the inhibition of citrate oxidation, occurring also *in vivo* (Buffa & Peters, 1949), is due to the 'jamming' effect of an enzymically synthesized fluoro-tricarboxylic acid in the Krebs tricarboxylic acid cycle. In support of this hypothesis, Buffa, Peters & Wakelin (1950) have isolated, from guinea-pig kidney homogenates treated with fluoroacetate, a tricarboxylic fraction, which is 'active' in preventing disappearance of added citrate. This active fraction is mainly citrate; it contains no fluoroacetate, but there is present a small amount of a F-compound which is chromatographically inseparable from the tricarboxylic acids.

We have tried to find the exact point of inhibition in the enzymes of the tricarboxylic acid cycle by determining the effect of the 'active' fractions upon aconitase (Johnson, 1939), isocitric dehydrogenase (Adler, Euler, Günther & Plass, 1939) and oxalosuccinic decarboxylase (Ochoa & Weiss-Tabori, 1948), obtained from rat and pig heart tissue. Tables 1, 2 and 3 show that the results were negative, even when amounts of 'active' fraction were used 80 times larger than those inhibiting citrate disappearance in the kidney homogenates.

All the evidence from experiments *in vivo* and *in vitro* (? mitochondrial homogenates) points to inhibition by the 'active' compound at either the

Table 1. *Rat heart aconitase*

Time (min.) ...	Citric acid (μmol.)	
Additions:	0	60
cis-Aconitate (5 μmol.)	0·21	3·90
cis-Aconitate + 'active' fraction	0·08	3·96
Citrate (5 μmol.)	4·90	4·34
Citrate + 'active' fraction	5·27	4·38

Table 2. *Pig heart isocitric dehydrogenase*

	$E_{340\ m\mu.}$ (max. value)
DL-isocitrate only	0·076–0·065
Same + 'active' fraction	0·075
Same + p-chloromercuribenzoic acid 1·33 × 10⁻⁴ M	0·004

Table 3. *Pig heart oxalosuccinic decarboxylase*

(CO₂ evolution from 10 μmol. oxalosuccinate in 30 min. at 13·5° C. Net values)

	CO₂ (μl.)
Enzyme alone	83
Enzyme + 'active' fraction	76
Enzyme + DL-isocitrate (control)	14

aconitase or isocitric dehydrogenase stage. Hence, we are led to the conclusion that the complete system has properties not present in its isolated enzyme components. Whether these be due to factors of organization or to missing components must be decided by further work.

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Nucleic Acids

Content and Distribution

Nucleic acids in an average human cell

DNA	
Coding sequences	~6 pg/cell a
Number of genes	3% of genomic DNA
Active genes	$0.5 1.0 \times 10^5$
	1.5×104
Total RNA	~10 50 pg/cell b
rRNAs	80 85% of total RNA
tRNAs, snRNAs, and low mol. wt. RNA	15 20% of total RNA
mRNAs	1 5% of total RNA
nuclear RNA	~14% of total RNA
Ratio of DNA:RNA in nucleus	~2:1
Number of mRNA molecules c	$0.2 1.0 \times 10^6$
Number of different mRNA species	$1.0 3.4 \times 10^4$
Low abundance mRNA (5 15 copies/cell)	11,000 different messages
Intermediate abundance mRNA (200 400 copies/cell)	500 different messages
High abundance mRNA (12,000 copies/cell)	<10 different messages
Abundance of each message for:	<0.004% of total mRNA
Low abundance mRNA (5 15 copies/cell)	<0.1% of total mRNA
Intermediate abundance mRNA (200 400 copies/cell)	3% of total mRNA
High abundance mRNA (12,000 copies/cell)	

a   30 – 60 μg/ml blood for human leukocytes.

b   1 – 5 μg/ml blood for human leukocytes.

c   Average size of mRNA molecule = 1930 bases.

RNA content of cells in culture

Type of cell	Total RNA (mRNA (μg/107 cells)	mRNA (μg/107 cells)
NIH/3T3 cells	75 200	1.5 4.0
HeLa cells	100 300	2 6
CHO cells	200 400	3 6

<< >>

Table of Content

UMRECHNUNGSTABELLEN

I. Conversiontable

Molecular weight (daltons)	1µg	1nmole
100	10 nmoles or 6×10^{15} molecules	0.1 µg
1,000	1 nmole or 6×10^{14} molecules	1 µg
10,000	100 pmoles or 6×10^{13} molecules	10 µg
20,000	50 pmoles or 3×10^{13} molecules	20 µg
30,000	33 pmoles or 2×10^{13} molecules	30 µg
40,000	25 pmoles or 1.5×10^{13} molecules	40 µg
50,000	20 pmoles or 1.2×10^{13} molecules	50 µg
60,000	17 pmoles or 10^{13} molecules	60 µg
70,000	14 pmoles or 8.6×10^{12} molecules	70 µg
80,000	12 pmoles or 7.5×10^{12} molecules	80 µg
90,000	11 pmoles or 6.6×10^{12} molecules	90 µg
100,000	10 pmoles or 6×10^{12} molecules	100 µg
120,000	8.3 pmoles or 5×10^{12} molecules	120 µg
140,000	7.1 pmoles or 4.3×10^{12} molecules	140 µg
160,000	6.3 pmoles or 3.8×10^{12} molecules	160 µg
180,000	5.6 pmoles or 3.3×10^{12} molecules	180 µg
200,000	5 pmoles or 3×10^{12} molecules	200 µg

II. Some useful nucleotide dimensions

1 cm of DNA ~ 3×10^6 nucleotides

Organism	Base pairs/ haploid genome	Base pairs/ diploid genome	Length/cell	Mass

Human	3×10^9	6×10^9	2 meters (diploid)	6 pg
Fly	1.65×10^8	3.3×10^8	100 cm (diploid)	0.3 pg
Yeast	1.35×10^7	2.7×10^7	10 cm (dolploid)	0.03 pg
E. coli	4.7×10^6	-	1.5 cm (diploid)	0.0045 pg
SV40	5×10^3	-	1.7 nm	0.000006 pg

III. Some useful cell dimensions

Organism	Dimensions	Volume
S. cerevisiae	5 µm	66 µm³
S. pombe	2 x 7 µm	22 µm³
Mammalian cell	10-20 µm	500-4,000 µm³
E. coli	1 x 3 µm	2 µm³
Mammalian mitochondrion	1 µm	0.5 µm³
Mammalian nucleus	5-10 µm	66-500 µm³
Plant chloroplast	1 x 4 µm	3 µm³
Bacteriophage lambda	50 nm (head only)	6.6×10^{-5} µm³
Ribosome	30 nm diameter	1.4×10^{-5} µm³
Globular monomeric protein	5 nm diameter	6.6×10^{-8} µm³

III. Some useful concentrations

Total cell protein concentration   Detergent soluble protein = 1-2 mg/ 10^7 mammalian cells or 100-200 mg/ ml for soluble proteins only

Specific protein concentrations
Nucleus (200 µm³):

    Abundant transcription factor   1 nM (100,000 copies/ nucleus)

    Rare transcription factor   10 pM (1,000 copies/ nucleus)

Serum   50-100 mg/ ml

IV. Some useful Conversiontables

Molar conversions for protein

100 pmol	µg
10,000 Da protein	1

100,000 Da protein	
	10

Protein/ DNA conversions

1 kb of DNA encodes 333 amino acids ~/= 3.7×10^4 Da

Protein	DNA
10,000 Da	270 dp
30,000 Da	810 dp
100,000 Da	2,7 dp

Nucleic acid content of a typical human cell

DNA per cell	~ 6 pg
Total RNA per cell	~ 10-30 pg
Proportion of total RNA in nucelus	~ 14%
DNA:RNA in nucleus	~2:1
Human genome size (haploid)	3.3×10^9 bp
Coding sequences/ genomic DNA	3%
Number of genes	$0.5-1 \times 10^5$
Active genes	1.5×10^4
mRNA molecules	$2 \times 10^5 - 1 \times 10^6$
Typical mRNA size	1900 nt

RNA distribution in a typical mammalian cell

RNA species	Relative amount
rRNA (28S, 18S, 5S)	80-85%
tRNAs, snRNAs, low MW species	15-20%
mRNAs	1-5%

RNA content in various cells and tissues

Source		Total RNA	mRNA (µg)
Cell cultures (10^7 cells)		30-500	0.3-25
	NIH/3T3	120	3
	HeLa	150	3
	COS-7	350	5
Mouse-developmental stages (per organism)	Unfertilized egg	0.43 ng	nd
	Oocyte	0.35 ng	nd

	2-cell	0.24 ng	nd
	8-16-cell	0.69 ng	nd
	32-cell	1.47 ng	nd
	13-day-old-embryo	450	13
Mouse tissue (100 mg)	Brain	120	5
	Heart	120	6
	Intestine	150	2
	Kidney	350	9
	Liver	400	14
	Lung	130	6
	Spleen	350	7

nd = not determined

Human blood*: cell, DNA, RNA, and protein content

	Leukocyes	Thrombocytes	Erythrocytes
Function	Immune response	Wound closing	O_2 & CO_2 transport
Cells per ml	$4-7 \times 10^6$	$3-4 \times 10^8$	5×10^9
DNA content	30-60 µg/ ml blood (6 pg/cell)		
RNa content	1-5 µg/ ml blood		
Hemoglobin content			~150 mg/ ml blood (30 pg/cell)
Plasma protein content		60-80 mg/ ml	

*From a healthy individual. The leukocyte concentration can vary from 2×106 per ml in cases of immunosuppression, to 40×10^6 during inflammation, to 500×10^6 during leukemia. The DNA and RNA content will vary accordingly.

zum Hauptmenü

EVIDENCE APPENDIX

ITEM NO. 28

Second Supplemental Declaration of Dr. Andrew E. Lorincz
(originally filed in co-pending application Serial No.
09/794,456) and cited by Appellant as Exhibit A in the Letter
filed May 25, 2007

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: James P. Elia)
)   Group Art Unit: 1646
Serial No.: 10/179,589)
)   Examiner: Elizabeth Kemmerer
Filed: June 25, 2002)
)
For: METHOD FOR GROWING)
    HUMAN ORGANS AND)
    SUBORGANS .)

SECOND SUPPLEMENTAL DECLARATION
OF ANDREW E. LORINCZ, M.D.

I, Andrew E. Lorincz, declare as follows:

1.   I reside at 16135 NW 243rd Way, High Springs, Florida 32643-3813.

2.   My Curriculum Vitae is attached was Exhibit A to my Declaration of November 8, 2004. Paragraph 3 of my Declaration and my Supplemental Declaration of June 5, 2006 provide additional information regarding my background and experience.

3.   I have read the Examiner's criticism contained in paragraph 11, commencing on page 7 and ending on page 9 of the March 7, 2007 Office Action regarding the conversion of dosages of plasmid cDNA to dosages of cells. Such paragraph is set forth in Second Supplemental Exhibit A attached hereto. Specifically, I note the Examiner's criticism bridging pages 7 and 8 regarding the above-mentioned conversion that:

1

...one of skill in the art would never think to attempt such an extrapolation. The unsound scientific basis for the conversion of plasmid DNA to cellular equivalents would be obvious to anyone trained in molecular biology.

4.   I have read and understood the disclosures of the above-referenced patent application at page 4, line 1 through page 5, line 14; at page 13, lines 3-10; at page 22, line 5 through page 24, line 15; and at page 26, line 3 through page 27, line 3. A copy of such disclosures is attached hereto as Second Supplemental Declaration Exhibit B.

I have also read and understood additional disclosures of the above-referenced patent application at page 9, lines 14-16; page 17, line 1 through page 20, line 8; page 21, lines 23 and 24; page 27, lines 1-3; page 28, lines 12-16; page 32, line 20 through page 39, line 19; and page 44, lines 8-17. A copy of such additional disclosures is attached hereto as Second Supplemental Declaration Exhibit C.

5.   I have read and understood Applicant's conversion for dosages of plasmid cDNA to equivalent corresponding dosages of cells set forth in attached Second Supplemental Exhibit D as it relates to Examples 18 and 17 of the specification, which are contained in Second Supplemental Declaration Exhibit C.

6.   In my opinion, the Examiner's criticism specifically delineated in Paragraph 3 above is not credible. Contrary to the Examiner's opinion, studies involving conversion of the average (mean) content of nucleic acids per cell in human marrow cells have been routinely conducted and accepted by skilled scientists for over 50 years. Three (3) publications illustrating the use of such well known conversion are included in the attached Second Supplemental Declaration Exhibit E. Note that in two of the publications, typical conversion results are set forth in tables, thereby eliminating the necessity to perform the actual calculation. Obviously, a sound scientific basis exists in the medical art for such conversions.

2

Further, those skilled in the art understand that DNA content is substantially consistent from tissues of any given species. Consequently, a skilled medical person relying on sound scientific bases at the time of the present invention would reasonably have understood how to extrapolate plasmid DNA to cells on a weight basis. Applicant's use of 40 pg as an average weight for nucleic acids in a human cell is fairly representative. Thus, I find Applicant's conversion set forth in the attached Second Supplemental Declaration Exhibit D to be consistent with the extrapolations set forth above and commonly used and relied upon by skilled persons in the medical art. Accordingly, the dosages specified in Examples 18 and 17 are sufficient to enable a person skilled in the medical art to convert dosages of plasmid DNA to corresponding dosages of genomic DNA within the context of Applicant's disclosed invention.

7.   Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: _4 – 1 9 – 0 7_

Andrew E. Lorincz, M.D.
Andrew E. Lorincz, M.D.

SECOND SUPPLEMENTAL
DECLARATION

EXHIBIT A

MARCH 2007
Office Action
Paragraph 11, pages 7-9

11.   Applicant admits on page 9 that Examples 17-19 employ nucleic acids, but asserts that

one skilled in the art reading the specification, which teaches that cells, i.e., stem cells (BMC's)

possess equivalent activity to genes (nucleic acids) and other genetic material in forming a new

artery (i.e., promote morphogenesis of an organ—artery), would be able to easily extrapolate the

number on a weight basis of mononuclear cells required to obtain equivalent results. According

to the method for extrapolation provided in the footnote to pages 10-11, 250 µg of plasmid DNA

(an amount described in Examples 17 and 18) divided by 40 pg, (asserted to be is the average

DNA content of a cell; the species of cell is not disclosed) equals 6.25×10^6, and therefore the

Examples 17 and 18 instruct the skilled artisan to use 6.25×10^6 cells. This argument is not

persuasive for several reasons. First, this method of converting plasmid DNA to cell equivalents

is not included in the specification as filed. This is important because one of skill in the art would

never think to attempt such an extrapolation. The unsound scientific basis for the conversion of

µg of plasmid DNA to cellular equivalents would be obvious to anyone trained in molecular

biology. One basic assumption of the recited conversion is that the 40 pg of cellular DNA comprises the same gene dosage a purified plasmid DNA. Every molecule of the postulated plasmid DNA comprises a copy of the VEGF cDNA. In contrast, VEGF coding sequences would comprise but one of 30-40 thousand genes in genomic DNA (at the time of filing, it was widely believed that the human genome comprised 100,000 genes). Therefore, one of skill in the art at the time of filing would not expect plasmid DNA and genomic DNA to be comparable on a per weight basis. Applicant's argument seems to view the living cell as little more than a container for DNA. The expression of the recombinant cDNA would be under control of the limited number of enhancer and promoter elements in the plasmid, as opposed the native control elements with the genome. Therefore, even equivalent gene doses would not be expected to yield equivalent amounts of gene product with a plasmid as opposed to a cell. Applicant's argument seems to view the living cell as little more than a container for DNA. Delivery of the genes to a target as recombinant DNA as opposed to native genes within a living cell are technically different processes; there is no basis for using one to guide the other. For example, with DNA one is concerned with chemical stability, efficiency of uptake, stable retention, and subsequent expression of the injected molecule into target cells, whereas with cells separate issues of formation of effective attachment to ECM and neighboring cells, short- and long-term viability, and responses to environmental cues arise. As evidence, one need look no further than the US Patent classification system. Methods of *in vivo* treatments involving whole live cells as opposed to nucleic acids are separately classified: class 424 subclass 93.1 (cells); class 514, subclass 44 (polynucleotides). These separate classifications indicate a different status in the art such that it is well known that cell therapy and gene therapy are not obvious variants of one

another. Therefore, contrary to Applicants assertion on page 9, the specification does not

describe any dosage of cells to use to promote artery growth.

SECOND SUPPLEMENTAL DECLARATION

EXHIBIT B

DISCLOSURES

EXHIBIT B

DISCLOSURES
APPLICATION SERIAL NO. 10/179,589

PAGE 4, LINE 1 – PAGE 5, LINE 14

Growth factors can be utilized to induce the growth of "hard tissue" or bone and "soft tissues" like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basis (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor an also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which an often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 13, LINES 3-10

Multifactorial and nonspecific cells (such as stem cells and germinal cells) can provide the necessary *in vivo* and *in vitro* cascade of genetic material once an implanted master control gene's transcription has been activated. Likewise, any host cell, cloned cell, cultured cell, or cell would work. Genetic switches (such as the insect hormone ecdysone) can be used to control genes inserted into humans and animals. These gene switches can also be used in cultured cells or other cells. Gene switches govern whether a gene is on or off making possible precise time of gene activity.

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in connection with any of the implant techniques of the invention, it is understood that a cell nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated

(taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth

factors (or other genetic material) are placed. Once the new muscle and blood vessels have grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

PAGE 26, LINE 3 – PAGE 27, LINE 3

Organs and/or tissues can be formed utilizing the patient's own cells. For example, a skin cell(s) is removed from the intraoral lining of a cheek. The cell is genetically screened to identify DNA damage or other structural and/or functional problems. Any existing prior art genetic screening technique can be utilized. Such methods can utilize lasers, DNA probes, PCR, or any other suitable device,. If the cell is damaged, a healthy undamaged cell is, if possible, identified and selected. If a healthy cell cannot be obtained, the damaged cell can be repaired by excision, alkylation, transition, or any other desired method. A growth factor(s) is added to the cell to facilitate dedifferentiation and then redifferentiation and morphogenesis into an organ or function specific tissue. Any machine known in the art can be used to check the genetic fitness of the organ and its stage of morphogenesis. A cell nutrient culture may or may not be utilized depending on the desired functional outcome (i.e., growth of an artery, of pancreatic Islet cells, of a heart, etc.) or other circumstances. Replantation can occur at any appropriate stage of

morphogenesis. The foregoing can be repeated without the patient's own cells if universal donor cells such as germinal cells are utilized. Germinal cells do not require a dedifferentiation. They simply differentiate into desired tissues or organs when properly stimulated. Similarly, the DNA utilized in the foreign procedure can come from the patient or from any desired source.

During reimplantation one of the patient's own cells is returned to the patient. During implantation, a cell not originally obtained from the patient is inserted on or in the patient.

In the example above, if germinal cells (and in some cases, stem cells) are utilized, a direct differentiation and morphogenesis into an organ can occur *in vivo, ex vivo,* or *in vitro.*

SECOND SUPPLEMENTAL DECLARATION

EXHIBIT C

ADDITIONAL DISCLOSURES

EXHIBIT C

DISCLOSURES
APPLICATION SERIAL NO. 10/179,589

PAGE 9, LINES 14-16

Morphogenesis or morphogenetics is the origin and evolution of morphological characters and is the growth and differentiation of cells and tissues during development.

PAGE 17, LINE 1 – PAGE 20, LINE 8

EXAMPLE 10

MSX-1 and MSX-2 are the homeobox genes that control the generation and growth of a tooth. A sample of skin tissue is removed from the patient and the MSX-1 and MSX-2 homeobox gene(s) are removed from skin tissue cells. The genes are stored in an appropriate nutrient culture medium.

BMP-2 and BMP-4 growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 and 102 degrees F.

MSX-1 and MSX-2 transcription factors are obtained which will initiate the expression of the MSX-1 and MSX-2 homeobox genes.

The MSX-1 and MSX-2 transcription factors, BMP-2 and MBP-4 bone morphogenic proteins, and MSX-1 and MSX-2 genes are added to the nutrient culture medium along with the living stem cells.

EXAMPLE 11

Example 10 is repeated except that the transcription factors bind to a receptor complex in the stem cell nucleus.

EXAMPLE 12

Example 10 is repeated except that the MSX-1 and MSX-2 transcription factors are not utilized. The transcription of the MSX-1 and MSX-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 13

Example 10 is repeated except that the stem cells are starved and the transcription of the MSX-1 and MSX-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 14

WT-1 and PAX genes are obtained from a sample of skin tissue removed from the patient. The genes are stored in an appropriate nutrient culture medium. PAX genes produce PAX-2 and other transcription factors.

BMP-7 and other kidney related BMP growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

The WT-1 and PAX genes, and BMP-7 and other kidney BMPS are added to the nutrient culture medium along with the living stem cells.

A primitive kidney germ is produced. The kidney germ is transplanted in the patient's body near a large artery. As the kidney grows, its blood supply will be derived from the artery.

EXAMPLE 15

The Aniridia gene is obtained from a sample of skin tissue removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

The Aniridia transcription factor and growth factor and the Aniridia gene are added to the nutrient culture medium along with the living stem cells.

A primitive eye germ is produced. The eye germ is transplanted in the patient's body near the optic nerve. As the eye grows, its blood supply will be derived from nearby arteries.

EXAMPLE 16

The Aniridia gene is obtained from a sample of skin tissue removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained and added to the nutrient culture medium.

An eye germ develops. A branch of the nearby maxillary artery is translocated to a position adjacent the eye germ to promote the development of the eye germ. The eye germ matures into an eye which receives its blood supply from the maxillary artery.

The term "cell nutrient culture" as used herein can include any or any combination of the following: the extracellular matrix; conventional cell culture nutrients; and/or, a cell nutrient such as a vitamin. As such, the cell nutrient culture can be two-dimensional, three-dimensional, or simply a nutrient, and is useful in promoting the processes of cellular dedifferentiation, redifferentiation, differentiation, growth, and development.

PAGE 21, LINES 23– 24

An organ, as used herein, consists of two or more kinds of tissues joined into one structure that has a certain task.

PAGE 27, LINES 1– 3

In the example above, if germinal cells (and in some cases, stem cells) are utilized a direct differentiation and morphogenesis into an organ can occur *in vivo*, *ex vivo*, or *in vitro*.

PAGE 28, LINES 12-16

Avascular necrosis can be corrected with the insertion of a gene(s) and/or growth factor or other genetic material in the body. For example, avascular necrosis is diagnosed near a joint space. VEGF or BMP genes, or VEFG or BMP growth factors produced by VEFG or BMP genes, respectively, or any other desired genetic based material can be inserted to regrow blood vessels and/or bone.

PAGE 32, LINE 20 – PAGE 39, LINE 19

EXAMPLE 17

A 36-year old Caucasian male experiences pain in his left leg. A medical examination reveals a damaged one-inch long section of a large artery in his left leg. The examination also reveals that this damaged section of the artery is nearly completely clogged with plaque and that the wall of the artery is weakened. The weakening in the arterial wall makes attempting to clean out the artery risky and also makes it risky to attempt to insert a stent in the artery.

Recombinant cDNA encoded to combine with a cell ribosome to produce the human growth factor VEGF is assembled into a eukaryotic expression plasmid. The recombinant

cDNA is from cDNA libraries prepared from HL60 leukemia cells and is known to cause the growth of arteries. The plasmid is maintained at a room temperature of 76 degrees F.

The clones are placed in 1.0 milliliters of a normal saline carrier solution at a room temperature of 76 degrees F. to produce a genetic carrier solution. The genetic carrier solution contains about 250 ug of the cDNA clones. A nutrient culture can, if desired, be utilized in conjunction with or in place of the saline carrier. Each clone is identical. If desired, only a single clone can be inserted in the normal saline carrier solution. The saline carrier solution comprises 0.09% by weight sodium chloride in water. A saline carrier solution is selected because it will not harm the DNA clone.

Two sites are selected for injection of the genetic carrier solution. While the selection of sites can vary as desired, the sites are selected at the lower end (the end nearest the left foot of the patient) of the damaged section of the artery so that the new arterial section grown, can, if necessary, be used to take the place of the damaged section of the artery in the event the damaged section is removed.

The first site is on the exterior wall of the artery on one side of the lower end of the damaged section of the artery. A containment system is placed at the first site.

The second site is inside the wall of the artery on the other side of the lower end of the artery.

The genetic carrier solution is heated to a temperature of 98.6 degrees F. 0.25 milliliters of the genetic carrier solution is injected into the containment system at the first site. 0.25 milliliters of the genetic carrier solution is injected at the second site inside the wall of the artery. Care is taken to slowly inject the genetic carrier solution to avoid entry of the solution into the artery such that blood stream will carry away the cDNA in the solution.

After two weeks, an MRI is taken which shows the patient's leg artery. The MRI reveals new growth at the first and second sites.

After four weeks, another MRI is taken which shows the patient's leg artery. The MRI shows that (1) at the first site, a new artery is growing adjacent the patient's original leg artery, and (2) at the second site, a new section of artery is growing integral with the original artery, i.e., at the second site the new section of artery is lengthening the original artery, much like inserting a new section of hose in a garden hose concentric with the longitudinal axis of the garden hose lengthens the garden hose.

After about eight to twelve weeks, another MRI is taken which shows that the new artery growing adjacent the patient's original artery has grown to a length of about one inch and has integrated itself at each of its ends with the original artery such that blood flows through the new section of artery. The MRI also shows that the new artery at the second site has grown to a length of one-half inch.

In any of the examples of the practice of the invention included herein, cell nutrient culture can be included with the gene, the growth factor, the extracellular matrix, or the environmental factors.

In any of the examples of the practice of the invention included herein, the concept of gene redundancy can be applied. For example, the Examples 1 to 14 concerning a tooth list the genes MSX-1 and MSX-2. These genes differ by only two base pairs. Either gene alone may be sufficient. A further example of redundancy occurs in growth factors. Looking at the Examples 10 to14, BMP4 or BMP2 alone may be sufficient. Redundancy can also be utilized in connection with transcription factors, extracellular matrices, environmental factors, cell nutrient culture, physiological nutrient cultures, vectors, promoters, etc.

One embodiment of the invention inserts genetic material (gene, growth factor, ECM, etc.) into the body to induce the formation of an organ. Similar inducing materials *ex vivo* into or onto a living cell in an appropriate physiological nurturing environment will also induce the growth of an organ. The VCSEL laser allows early detection in a living cell of a morphogenic change indicating that organ formation has been initiated. With properly time transplantation, organ growth completes itself.

During the *ex vivo* application of the invention, a gene and/or growth factor is inserted into a cell or a group of cells; an ECM or environmental factor(s) are placed around and in contact with a cell or group of cells; or, genetic material is inserted into a subunit of a cell to induce organ growth. An example of a subunit of a cell is an enucleated cell or a comparable artificially produced environment. In *in vivo* or *ex vivo* embodiments of the invention to induce the growth of an organ, the genes, growth factors, or other genetic material, as well as the environmental factors or cells utilized, can come from any desired source.

EXAMPLE 18

Genetically produced materials are inserted in the body to cause the body to grow, reproduce, and replace *in vivo* a clogged artery in the heart. This is an example of site-specific gene expression. A plasmid expression vector containing an enhancer/promoter is utilized to aid in the transfer of the gene into muscle cells. The enhancer is utilized to drive the specific expression of the transcriptional activator. After the enhancer drives the expression of the transcriptional activator, the transcriptional activator transactivates the muscle/artery genes. Saline is used as a carrier. Cardiac muscle can take up naked DNA injection intramuscularly.

Injecting plasmid DNA into cardiac (or skeletal) muscle results in expression of the transgene in cardiac myocytes for several weeks or longer.

Readily available off-the-shelf (RAOTS) cDNA clones for recombinant human VEFG165, isolated from cDNA libraries prepared from HL60 leukemia cells, are assembled in a RAOTS expression plasmid utilizing 736bp CMV promoter/enhancer to drive VEGF expression. Other RAOTS promoters can be utilized to drive VEGF expression for longer periods of time. Other RAOTS recombinant clones of angiogenic growth factors other than VEGF can be utilized, for example, fibroblast growth factor family, endothelial cell growth factor, etc. Downstream from the VEGF cDNA is an SV40 polyadenylation sequence. These fragments occur in the RAOTS pUC118 vector, which includes an Escherichia coli origin of replication and the Beta lactamase gene for ampicillin resistance.

The RAOTS construct is placed into a RAOTS 3 ml syringe with neutral pH physiologic saline at room temperature (or body temperature of about 73 degrees C). The syringe has a RAOTS 27 gauge needle.

Access to the cardiac muscle is gained by open-heart surgery, endoscopic surgery, direct injection of the needle with incision, or by any other desired means. The cardiac muscle immediately adjacent a clogged artery is slowly injected with the RAOTS construct during a five second time period. Injection is slow to avoid leakage through the external covering of muscle cells. About 0.5 ml to 1.0 ml (milliliter) of fluid is injected containing approximately 500 ug phVEGF165 in saline (N=18). The readily available off-the-shelf cDNA clones cause vascular growth which automatically integrates itself with the cardiac muscle. Anatomic evidence of collateral artery formation is observed by the 30th day following injection to the RAOTS construct. One end of the artery integrates itself in the heart wall to receive blood from the heart.

The other end of the artery branches into increasingly smaller blood vessels to distribute blood into the heart muscle. Once the growth of the new artery is completed, the new artery is left in place in the heart wall. Transplantation of the new artery is not required.

Blood flow through the new artery is calculated in a number of ways. For example, Doppler-derived flow can be determined by electromagnetic flowmeters (using, for example, a Doppler Flowmeter sold by Parks Medical Electronic of Aloha, Oregon) both *in vitro* and *in vivo*. Also, RAOTS angiograms or any other readily available commercial devices can be utilized.

VEGF gene expression can be evaluated by readily available off-the-shelf polymerase chain reaction (PCR) techniques.

If controls are desired, the plasmid pGSVLacZ containing a nuclear targeted Beta-galactosidase sequence coupled to the simian virus 40 early promoter can be used. To evaluate efficiency, a promoter-matched reporter plasmid, pCMV Beta (available from Clontech of Palo Alto, California), which encodes Beta-galactosidase under control of CMV promoter/enhancer, can be utilized. Other RAOTS products can be utilized if desired.

EXAMPLE 19

A patient, a forty-year old African-American female in good health, has been missing tooth number 24 for ten years. The space in her mouth in which her number 24 tooth originally resided is empty. All other teeth except tooth number 24 are present in the patient's mouth. The patient desires a new tooth in the empty "number 24" space in her mouth.

A full thickness mucoperiosteal flap surgery is utilized to expose the bone in the number 24 space. A slight tissue reflection into the number 23 tooth and number 25 tooth areas is carried out to insure adequate working conditions.

A Midwest Quietair handpiece (or other off-the-shelf handpiece) utilizing a #701 XXL bur (Dentsply Midwest of Des Plaines, Illinois) (a #700, #557, #558, etc. bur can be utilized if desired) is used to excavate an implant opening or site in the bone. The implant opening is placed midway between the roots of the number 23 and number 25 teeth. The opening ends at a depth which is about fifteen millimeters and which approximates the depth of the apices of the roots of the number 23 and number 25 teeth. Care is taken not to perforate either the buccal or lingual wall of the bone. In addition, care is taken not to perforate or invade the periodontal ligament space of teeth numbers 23 and 25.

An interrupted drilling technique is utilized to avoid overheating the bone when the #701XXL bur is utilized to form the implant opening. During a drilling sequence, the drill is operated in five-second increments; and the handpiece is permitted to stall. Light pressure and a gentle downward stroke are utilized.

PAGE 44, LINES 8–17

EXAMPLE 35

Example 17 is repeated except that the patient is a 55-year old Caucasian male, and the genetic carrier solution is injected into two sites in the coronary artery of the patient. The first site is on the exterior wall on one side of the artery. The second site is inside the wall of the artery on the other side of the artery. A section of the artery is damaged, is partially blocked, and has a weakened wall. The first and second sites are each below the damaged section of the

artery. Similar results are obtained, i.e., a new section of artery grows integral with the original

artery, and a new section of artery grows adjacent the original artery. The new section of artery

has integrated itself at either end with the original artery so that blood flows through the new

section of artery.

SECOND SUPPLEMENTAL DECLARATION

ENTITLED

COMMON

EXHIBIT D

CONVERSION

The conversion for dosages of nucleic acids to corresponding dosages of cells was conducted as follows. Examples 18 and 17 specified dosages of 500 micrograms (ug) and 250 ug, respectively. The weight of nucleic acids of an average cell was considered to equal 40 picograms (pg). The described dosages of 250 and 500 ug when converted to pg by multiplying by 10^6 equals 250×10^6 pg and 500×10^6 pg. Since nucleic acids of an average cell have an average weight of 40 pg, a conversion is made by dividing 250×10^6 and 500×10^6 by 40 to arrive at the equivalent cell dosages, which are 6.25×10^6 and 12.5×10^6, respectively.

SECOND SUPPLEMENTAL DECLARATION

EXHIBIT E

PUBLICATIONS (3)

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

Studies on the Average Content of Nucleic Acids in Human Marrow Cells. By J. N. DAVIDSON, I. LESLIE and J. C. WHITE. (*From the Department of Biochemistry, University of Glasgow, and the Department of Pathology, Postgraduate Medical School of London*)

In extension of previously reported analyses of the deoxyribonucleic acid phosphorus (DNAP) and ribonucleic acid phosphorus (RNAP) content of aspirated human bone marrow (Davidson, Leslie & White, 1947, 1948), we now report a modification involving enumeration of the nucleated cell content of the samples analysed. Results are expressed in terms of DNAP and RNAP per cell (Table 1), and are average values for the growing and adult cell populations of the analysed samples. The recent results of Vendrely & Vendrely (1948, 1949) and of Mirsky & Ris (1949) suggest a striking constancy in the DNAP content of normal cell nuclei from the tissues of any given species, and our figures for DNAP are of the same order as those quoted by the Vendrelys for human liver nuclei.

There is no significant difference between the means for the normal and the leukaemic series, either as a whole, or considering only acute leukaemia prior to therapy.

A small series of 6 cases of iron-deficiency anaemia has not shown significant variation of the mean DNAP and RNAP per cell from normal.

Results obtained from cases of pernicious and other megaloblastic anaemias are shown in Tables 2 and 3.

It must be noted clearly that the group under therapy cannot be considered as returned to normal, either as regards blood picture, marrow cytology or adequacy of therapy. The significant fall in RNAP from that in the group prior to therapy parallels the general increase in maturity of the marrow under therapy. Cases fully treated and returned to normal are under investigation.

Table 1

Normal human marrow

Values of Nucleic Acid Phosphorus (NAP) in μg. $\times 10^{-7}$ per cell

	DNAP 18 obs. on 16 individuals	RNAP 20 obs. on 18 individuals	Ratio RNAP/ DNAP
Mean	8·54	6·33	0·75
s.e. of obs.	2·89	3·03	0·326
Observed range	4·0–15·0	2·1–13·5	0·43–1·9

Marrow from cases of leukaemia of various types, before and during therapy

	28 obs. on 15 cases	24 obs. on 12 cases	
Mean	8·75	7·59	0·90
s.e. of obs.	3·05	3·72	0·30
Observed range	3·9–17·4	2·0–17·4	0·3–1·8

Table 2. *Cases of pernicious anaemia and other megaloblastic anaemias*

NAP in μg. $\times 10^{-7}$ per cell

		DNAP	RNAP	Ratio DNAP/RNAP
Group as a whole	Mean	28 obs. on 12 cases 12·6	10·9	28 obs. on 13 cases 0·87
	s.e.	4·56	5·03	0·27
	Observed range	6·6–22·8	2·3–25·1	0·35–1·5
Group prior to therapy	Mean	12 obs. on 12 cases 12·57	11 obs. on 11 cases 13·38	12 obs. on 12 cases 1·06
	s.e.	4·17	5·19	0·249
	Observed range	8·1–22·8	7·5–25·1	0·69–1·5
Group during the course of therapy	Mean	17 obs. on 8 cases 12·63	15 obs. on 8 cases 9·09	16 obs. on 9 cases 0·73
	s.e.	4·36	4·21	0·198
	Observed range	6·6–18·8	2·3–17·6	0·35–1·0

Table 3. *t test of significance between means*

		DNAP	RNAP	Ratio RNAP/DNAP
Megaloblastic series as a whole compared with normal series	P Degrees of freedom	<0·001 44 Highly significant	<0·001 44 Highly significant	0·2–0·1 46 Not significant
Megaloblastic series before therapy compared with normal	P Degrees of freedom	0·01–0·001 28 Highly significant	<0·001 29 Highly significant	0·01–0·001 30 Highly significant
Megaloblastic series during therapy compared with normal	P Degrees of freedom	0·01–0·001 33 Highly significant	0·05–0·02 33 Significant	0·8–0·7 34 Not significant
Megaloblastic series before and during therapy compared	P Degrees of freedom	0·7–0·6 27 Not significant	0·05–0·02 24 Significant	<0·001 26 Highly significant

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Fluoroacetate Poisoning and 'Jamming' of the Tricarboxylic Acid Cycle; Mode of Action of an 'Active' Fluoro Compound Synthesized via this Cycle. By P. BUFFA, W. D. LOTSPEICH, R. A. PETERS and R. W. WAKELIN. (*Department of Biochemistry, University of Oxford*)

So far no isolated enzyme has been inhibited by fluoroacetate. The hypothesis has been advanced by Liébecq & Peters (1949) (see also Martius, 1949) that the inhibition of citrate oxidation, occurring also *in vivo* (Buffa & Peters, 1949), is due to the 'jamming' effect of an enzymically synthesized fluoro-tricarboxylic acid in the Krebs tricarboxylic acid cycle. In support of this hypothesis, Buffa, Peters & Wakelin (1950) have isolated, from guinea-pig kidney homogenates treated with fluoroacetate, a tricarboxylic fraction, which is 'active' in preventing disappearance of added citrate. This active fraction is mainly citrate; it contains no fluoroacetate, but there is present a small amount of a F-compound which is chromatographically inseparable from the tricarboxylic acids.

We have tried to find the exact point of inhibition in the enzymes of the tricarboxylic acid cycle by determining the effect of the 'active' fractions upon aconitase (Johnson, 1939), isocitric dehydrogenase (Adler, Euler, Günther & Plass, 1939) and oxalo-succinic decarboxylase (Ochoa & Weiss-Tabori, 1948), obtained from rat and pig heart tissue. Tables 1, 2 and 3 show that the results were negative, even when amounts of 'active' fraction were used 80 times larger than those inhibiting citrate disappearance in the kidney homogenates.

All the evidence from experiments *in vivo* and *in vitro* (? mitochondrial homogenates) points to inhibition by the 'active' compound at either the

Table 1. *Rat heart aconitase*

Time (min.) ...	Citric acid (µmol.)	
	0	60
Additions:		
cis-Aconitate (5 µmol.)	0·21	3·90
cis-Aconitate + 'active' fraction	0·08	3·96
Citrate (5 µmol.)	4·90	4·34
Citrate + 'active' fraction	5·27	4·38

Table 2. *Pig heart isocitric dehydrogenase*

	$E_{340\,m\mu}$ (max. value)
DL-isocitrate only	0·076–0·065
Same + 'active' fraction	0·075
Same + p-chloromercuribenzoic acid 1·33 × 10⁻⁴ M	0·004

Table 3. *Pig heart oxalosuccinic decarboxylase*

(CO_2 evolution from 10 µmol. oxalosuccinate in 30 min. at 13·5° C. Net values)

Enzyme	CO_2 (µl.)
Enzyme alone	83
Enzyme + 'active' fraction	76
Enzyme + DL-isocitrate (control)	14

aconitase or isocitric dehydrogenase stage. Hence, we are led to the conclusion that the complete system has properties not present in its isolated enzyme components. Whether these be due to factors of organization or to missing components must be decided by further work.

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Nucleic Acids

Content and Distribution

Nucleic acids in an average human cell

DNA	
Coding sequences	~6 pg/cella
Number of genes	3% of genomic DNA
Active genes	$0.5 1.0 \times 10^5$
	1.5×104
Total RNA	
rRNAs	~10 50 pg/cellb
tRNAs, snRNAs, and low mol. wt. RNA	80 85% of total RNA
mRNAs	15 20% of total RNA
nuclear RNA	1 5% of total RNA
Ratio of DNA:RNA in nucleus	~14% of total RNA
Number of mRNA moleculesc	~ 2:1
Number of different mRNA species	$0.2 1.0 \times 10^6$
Low abundance mRNA (5 15 copies/cell)	$1.0 3.4 \times 10^4$
Intermediate abundance mRNA (200 400 copies/cell)	11,000 different messages
High abundance mRNA (12,000 copies/cell)	500 different messages
Abundance of each message for:	<10 different messages
Low abundance mRNA (5 15 copies/cell)	<0.004% of total mRNA
Intermediate abundance mRNA (200 400 copies/cell)	<0.1% of total mRNA
High abundance mRNA (12,000 copies/cell)	3% of total mRNA

a   30 – 60 µg/ml blood for human leukocytes.

b   1 – 5 µg/ml blood for human leukocytes.

c   Average size of mRNA molecule = 1930 bases.

RNA content of cells in culture

Type of cell	Total RNA (mRNA (µg/107 cells))	mRNA (µg/107 cells)
NIH/3T3 cells	75 200	1.5 4.0
HeLa cells	100 300	2 6
CHO cells	200 400	3 6

<>

Table of Content

UMRECHNUNGSTABELLEN

I. Conversiontable

Molecular weight (daltons)	1µg	1nmole
100	10 nmoles or 6×10^{15} molecules	0.1 µg
1,000	1 nmole or 6×10^{14} molecules	1 µg
10,000	100 pmoles or 6×10^{13} molecules	10 µg
20,000	50 pmoles or 3×10^{13} molecules	20 µg
30,000	33 pmoles or 2×10^{13} molecules	30 µg
40,000	25 pmoles or 1.5×10^{13} molecules	40 µg
50,000	20 pmoles or 1.2×10^{13} molecules	50 µg
60,000	17 pmoles or 10^{13} molecules	60 µg
70,000	14 pmoles or 8.6×10^{12} molecules	70 µg
80,000	12 pmoles or 7.5×10^{12} molecules	80 µg
90,000	11 pmoles or 6.6×10^{12} molecules	90 µg
100,000	10 pmoles or 6×10^{12} molecules	100 µg
120,000	8.3 pmoles or 5×10^{12} molecules	120 µg
140,000	7.1 pmoles or 4.3×10^{12} molecules	140 µg
160,000	6.3 pmoles or 3.8×10^{12} molecules	160 µg
180,000	5.6 pmoles or 3.3×10^{12} molecules	180 µg
200,000	5 pmoles or 3×10^{12} molecules	200 µg

II. Some useful nucleotide dimensions

1 cm of DNA ~ 3×10^6 nucleotides

Organism	Base pairs/ haploid genome	Base pairs/ diploid genome	Length/cell	Mass

Human	3×10^9	6×10^9	2 meters (diploid)	6 pg
Fly	1.65×10^8	3.3×10^8	100 cm (diploid)	0.3 pg
Yeast	1.35×10^7	2.7×10^7	10 cm (dolploid)	0.03 pg
E. coll	4.7×10^6	-	1.5 cm (diploid)	0.0045 pg
SV40	5×10^3	-	1.7 nm	0.000006 pg

III. Some useful cell dimensions

Organism	Dimensions	Volume
S. cerevislae	5 µm	66 µm³
S. pombe	2 x 7 µm	22 µm³
Mammalian cell	10-20 µm	500-4,000 µm³
E. coll	1 x 3 µm	2 µm³
Mammalian mitochondrion	1 µm	0.5 µm³
Mammalian nucleus	5-10 µm	66-500 µm³
Plant chloroplast	1 x 4 µm	3 µm³
Bacteriophage lambda	50 nm (head only)	6.6×10^{-5} µm³
Ribosome	30 nm diameter	1.4×10^{-5} µm³
Globular monomeric protein	5 nm diameter	6.6×10^{-8} µm³

III. Some useful concentrations

Total cell protein concentration — Detergent soluble protein = 1-2 mg/ 10^7 mammalian cells or 100-200 mg/ ml for soluble proteins only

Specific protein concentrations

Nucleus (200 µm³):

Abundant transcription factor — 1 nM (100,000 copies/ nucleus)

Rare transcription factor — 10 pM (1,000 copies/ nucleus)

Serum — 50-100 mg/ ml

IV. Some useful Conversiontables

Molar conversions for protein

100 pmol	µg
10,000 Da protein	1

100,000 Da protein	10

Protein/ DNA conversions

1 kb of DNA encodes 333 amino acids ~/= 3.7×10^4 Da

Protein	DNA
10,000 Da	270 dp
30,000 Da	810 dp
100,000 Da	2,7 dp

Nucleic acid content of a typical human cell

DNA per cell	~ 6 pg
Total RNA per cell	~ 10-30 pg
Proportion of total RNA in nucelus	~ 14%
DNA:RNA in nucleus	~2:1
Human genome size (haploid)	3.3×10^9 bp
Coding sequences/ genomic DNA	3%
Number of genes	$0.5-1 \times 10^5$
Active genes	1.5×10^4
mRNA molecules	$2 \times 10^5 - 1 \times 10^6$
Typical mRNA size	1900 nt

RNA distribution in a typical mammalian cell

RNA species	Relative amount
rRNA (28S, 18S, 5S)	80-85%
tRNAs, snRNAs, low MW species	15-20%
mRNAs	1-5%

RNA content in various cells and tissues

Source		Total RNA	mRNA (µg)
Cell cultures (10^7 cells)		30-500	0.3-25
	NIH/3T3	120	3
	HeLa	150	3
	COS-7	350	5
Mouse-developmental stages (per organism)	Unfertilized egg	0.43 ng	nd
	Oocyte	0.35 ng	nd

	2-cell	0.24 ng	nd
	8-16-cell	0.69 ng	nd
	32-cell	1.47 ng	nd
	13-day-old-embryo	450	13
Mouse tissue (100 mg)			
	Brain	120	5
	Heart	120	6
	Intestine	150	2
	Kidney	350	9
	Liver	400	14
	Lung	130	6
	Spleen	350	7

nd = not determined

Human blood*: cell, DNA, RNA, and protein content

Function	Leukocyes Immune response	Thrombocytes Wound closing	Erythrocytes O_2 & CO_2 transport
Cells per ml	$4-7 \times 10^6$	$3-4 \times 10^8$	5×10^9
DNA content	30-60 µg/ ml blood (6 pg/cell)		
RNa content	1-5 µg/ ml blood		
Hemoglobin content			~150 mg/ ml blood (30 pg/cell)
Plasma protein content		60-80 mg/ ml	

*From a healthy individual. The leukocyte concentration can vary from 2×106 per ml in cases of immunosuppression, to 40×10^6 during inflammation, to 500×10^6 during leukemia. The DNA and RNA content will vary accordingly.

zum Hauptmenü

EVIDENCE APPENDIX

ITEM NO. 29

**Dohmann, et al., 2005 publication in <u>Circulation</u>, entitled,
"Transendocardial, Autologous Bone Marrow Mononuclear
Cell Injection in Ischemic Heart Failure"
cited in Examiner's Answer, dated November 28, 2007
in co-pending application Serial No. 09/794,456**

(also attached hereto as Exhibit F)

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Transendocardial Autologous Bone Marrow Mononuclear Cell Injection in Ischemic Heart Failure

Postmortem Anatomicopathologic and Immunohistochemical Findings

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▶ Abstract

Background— Cell-based therapies for treatment of ischemic heart disease are currently under investigation. We previously reported the results of a phase I trial of transendocardial injection of autologous bone marrow mononuclear (ABMM) cells in patients with end-stage ischemic heart disease. The current report focuses on postmortem cardiac findings from one of the treated patients, who died 11 months after cell therapy.

Methods and Results— Anatomicopathologic, morphometric, and immunocytochemical findings from the anterolateral ventricular wall (with cell therapy) were compared with findings from the interventricular septum (normal perfusion and no cell therapy) and from the inferoposterior ventricular wall (extensive scar tissue and no cell therapy). No signs of adverse events were found in the cell-injected areas. Capillary density was significantly higher ($P<0.001$) in the anterolateral wall than in the previously infarcted tissue in the posterior wall. The prominent vasculature of the anterolateral wall was associated with hyperplasia of pericytes, mural cells, and adventitia. Some of these cells had acquired cytoskeletal elements and contractile proteins (troponin, sarcomeric α-actinin, actinin), as well as the morphology of cardiomyocytes, and appeared to have migrated toward adjacent bundles of cardiomyocytes.

Conclusions— Eleven months after treatment, morphological and immunocytochemical analysis of the sites of ABMM cell injection showed no abnormal cell growth or tissue lesions and suggested that an active process of angiogenesis was present in both the fibrotic cicatricial tissue and the adjacent cardiac muscle. Some of the pericytes had acquired the morphology of cardiomyocytes, suggesting long-term sequential regeneration of the cardiac vascular tree and muscle.

Key Words: angiogenesis • stem cells • heart failure • revascularization • ischemia

▶ Introduction

The role of cell-based therapy for the treatment of ischemic heart disease is currently under investigation. In view of the myocardium's limited capacity to regenerate spontaneously after an ischemic injury, the therapeutic use of exogenous progenitor cells has recently gained increasing interest. In vitro demonstration of functional cardiomyocyte differentiation from bone marrow–derived progenitor cells[1,2] has prompted in vivo studies in animal models, and promising results have been obtained in the repair and regeneration of acute and chronic cardiac muscle lesions. Several types of progenitor cells have been used in experimental models, including bone marrow–derived endothelial and blood cell progenitors, as well as bone marrow mesenchymal progenitors.[3–6]

In humans, similar attempts have been made with surgical, intracoronary, or transendocardial introduction of bone marrow–derived cells to improve cardiac lesions.[7,8] Our group recently reported the results of the first phase I human trial of transendocardial injection of autologous bone marrow mononuclear (ABMM) cells in patients with end-stage ischemic heart disease.[9] We observed a significant increase in perfusion, contractility of ischemic myocardial segments, and functional capacity of the cell-injection recipients. This report presents postmortem cardiac findings from one of these patients.

▶ Case Report

The patient was a 55-year-old man with ischemic cardiomyopathy and 2 previous myocardial infarctions (in 1985 and 2000). He began to have symptoms of congestive heart failure 2 years before study enrollment. One year before enrollment, the patient had an ischemic stroke with mild residual right hemiparesis

and resultant episodes of chi ̲ ̲ic tonic-clonic seizures. His risk factors for coronary
artery disease included diabetes mellitus type II, hypertension, and
hypercholesterolemia.

The patient's functional capacity was evaluated at baseline by means of a ramp treadmill protocol[10] with a peak maximal oxygen consumption ($\dot{V}O_2$max) of 15.9 mL · kg^{-1} · min^{-1} and a workload of 4.51 metabolic equivalents (METs). A baseline single-photon-emission computed tomography (SPECT) perfusion study showed a partially reversible perfusion defect in the anterolateral wall, a fixed perfusion defect (scar) in the inferior and posterior walls, and normal perfusion in the septal wall.

Cardiac catheterization revealed a left ventricular ejection fraction of 11%, a 70% ostial and an 85% middle stenosis of the left anterior descending (LAD) coronary artery, an 80% proximal lesion of the left circumflex (LCx) coronary artery, and total occlusion of the first obtuse marginal artery and right coronary artery. The distal segments of the LAD and LCx were diffusely diseased. Owing to the severity and extent of the patient's coronary disease, he was not considered a candidate for surgical or interventional procedures. At enrollment in our study, he was in New York Heart Association (NYHA) functional class III and Canadian Cardiovascular Society (CCS) angina class III. His serum C-reactive protein level, complete blood count, creatine kinase level, and troponin level were normal at baseline.

The patient received a total of 3x10^7 ABMM cells (the Table) that had been harvested 2 hours before the procedure. With the guidance of electromechanical mapping,[11,12] the cells were injected transendocardially into the anterolateral wall of the left ventricle. No periprocedural complications were observed.

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Phenotype and Functional Characterization of 3x10^7 Cells Injected via a Transendocardial Route[*]

Noninvasive follow-up evaluation was performed 2 and 6 months after cell therapy. Invasive follow-up evaluation, with cardiac catheterization, was performed at 4 months and revealed no change in coronary anatomy. Symptomatic and functional improvements were noted because the patient returned to NYHA and CCS class I. Holter monitoring showed no malignant ventricular arrhythmias, and signal-averaged ECG parameters remained stable. There was no change in the patient's medications after cell therapy. There was no change in the global ejection fraction or left

ventricular volume on echocardiography. The wall-motion index score (on 2-dimensional echocardiography) improved from 1.94 to 1.65 as contractility increased in 5 segments adjacent to the injected area. Myocardial perfusion, as assessed by SPECT, improved in the anterolateral wall. Mechanical data derived from SPECT showed improvements in regional ejection fraction, wall motion, and thickening. In addition, during ramp treadmill testing, the $\dot{V}O_2$max increased from 15.8 to 25.2 mL $\cdot$ kg^{-1} $\cdot$ min^{-1}, and the METs increased from 4.51 to 7.21 at 2 months. At 6-month follow-up testing, the $\dot{V}O_2$max reached 31.6 mL $\cdot$ kg^{-1} $\cdot$ min^{-1}, and the METs was 9.03.

From 6 to 11 months after the cell injection procedure, the patient's cardiovascular condition remained stable. At 11 months, however, he had a tonic-clonic seizure at home and was found in cardiopulmonary arrest by family members.

▶ Methods

After signed, informed consent was given by the family, an autopsy was performed, including morphological and immunocytochemical analysis of the heart. This report presents the anatomicopathologic findings about the infarcted areas of the anterolateral ventricular wall, which were the areas that had received bone marrow cell injections. The histological findings from this region were compared with findings from within the interventricular septum (which had normal perfusion in the central region and no cell therapy) and findings from the previously infarcted inferoposterior ventricular wall (which had extensive scarring and no cell therapy).

Immunocytochemical analysis of paraffin sections was performed with antibodies against factor VIII–related antigen (A0082, Dako), vimentin (M0725, Dako), smooth muscle α-actin (M0851, Dako), and CD34 and Ki-67 (NCL-L-End and NCL-Ki67MM1 respectively, Novocastra). Antibodies were reacted with Dako's EnVision+ System/HRP, with diaminobenzidine as a chromogen. Frozen sections were fixed, permeated with acetone, and incubated with antibodies for troponin T (T6277, Sigma), smooth muscle α-actin, sarcomeric actinin (A7811, Sigma), and desmin (D1033, Sigma). Antibodies were revealed with anti-mouse or anti-rabbit IgG, F(ab)$_2$ fragment, conjugated to fluorescein isothiocyanate (1814192 and 1238833, respectively, Boehringer-Mannheim), and counterstained with a 0.1‰ solution of Evans blue dye (Merck).

Capillary density was monitored by using computerized image analysis (Image-Pro Plus, MediaCybernetics) of randomly selected fields in sections stained with hematoxylin and reacted with antibody for factor VIII–related antigen (n=108) and randomly selected fields in sections reacted with antibodies for smooth muscle α-actin (n=96). Transverse sections of capillaries identified by staining

for factor VIII and pericyte-containing capillaries identified by staining for smooth muscle α-actin were quantified separately. Results were expressed as the mean number of capillaries per square millimeter in the case of factor VIII–stained slides or the number of capillaries containing pericytes in α-smooth-muscle-actin–stained slides. Larger vessels identified by a continuous wall of smooth muscle actin–positive mural cells were excluded. Differences between the anterolateral, septal, and posterior walls were assessed with Kruskal-Wallis ANOVA and the Student-Newman-Keuls method for pairwise multiple comparison. Results were considered significant if P was <0.05.

Evaluation of the capillary density inside the fibrotic areas within the cell-treated anterolateral wall versus the nontreated posterior wall was performed in 40 selected fields inside the fibrotic scars, excluding the regions containing cardiomyocytes. Microscope fields (at ×100) of factor VIII–stained slides were digitized, and the number of transverse sections of capillaries per square millimeter of fibrotic zones was assessed. Differences between the treated infarcted zones and the nontreated fibrotic wall were assessed by the Mann-Whitney rank-sum test. Results were considered significant if P was <0.05.

▶ Results

Anatomopathologic Findings

The heart weighed 765 g. There was severe arteriosclerosis with subocclusive calcified atheromata in all coronary arteries, calcification of the pulmonary artery, and moderate atheromatosis of the aorta. The heart cavities were dilated, with hypertrophic walls. There was no evidence of any acute injury or of lesions that could be related to cell injections. A generalized, homogeneous endocardial opacification, affecting all the cardiac internal surfaces, was identified on histological examination as diffuse fibroelastic hyperplasia of the endocardium. Minute focal and punctate scars were observed, mainly in the posterior and anterolateral walls.

The apical zone was thinned and fibrotic. The posterior and apical regions had dense, fibrotic, well-circumscribed scars that separated cardiomyocyte bundles. The septal wall exhibited focal scars interspersed with cardiac fibers in the regions adjacent to the anterior and posterior ventricular walls, but it was devoid of fibrosis in the central region.

The anterolateral ventricular wall that received cell injections had elongated, irregular, and parallel reddish areas throughout. In the same wall, in adjacent regions that did not receive injections, the density and morphology of the fibrotic scars were similar to those of the posterior wall, suggesting that no overt differences were present among the different infarcted areas before cell injections.

Morphometric Analysis

The capillary density was significantly higher in the areas of the anterolateral ventricular walls that received cell injections than in the previously infarcted posterior wall ($P<0.0001$) (Figure 1A). The median capillary density in the anterolateral wall was apparently similar to that in the septal wall. However, the broad dispersion of the septal wall data, which may have been due to fibrotic areas in regions close or adjacent to the ventricular walls, generated a statistically significant difference between these 2 groups.

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Figure 1. Number of capillaries per mm^2 in anterolateral, posterior, and septal walls of studied heart. A, Anti-factor VIII–associated antigen counterstained with hematoxylin. B, Anti-smooth muscle ▬-actin antigen counterstained with hematoxylin. C, Capillaries reacted with anti-factor VIII–associated antigen inside fibrotic areas only in anterolateral and posterior walls. (n=108 microscope fields for A; 96 microscope fields for B; and 40 microscopic fields for C.) Differences were statistically significant among all groups in pairwise comparisons ($P<0.05$, Newman-Keuls method) for A and B. Differences were significantly different ($P<0.05$) between anterolateral and posterior walls in Mann-Whitney rank-sum test for C.

The density of capillaries that contained smooth muscle α-actin–positive cells within their walls was also assessed (Figure 1B). The number of such vessels was higher in the anterolateral wall than in the septal and posterior walls ($P<0.0001$). Larger vessels identified by a continuous wall of smooth muscle α-actin–positive mural cells were not included in these analyses. The capillary density was significantly higher within fibrotic areas of the anterolateral wall than within fibrotic areas of the posterior wall ($P<0.0001$) (Figure 1C).

Histological Findings

The anterolateral wall showed irregular, pale regions of fibrotic tissue intercalated with dark regions of cardiac muscle arranged in roughly parallel, interspersed bands, perpendicular to the ventricular wall plane (Figure 2A). No abnormal cell organization, growth, or differentiation or signs of previous focal necrosis, inflammatory reactions, or tissue repair were found in the region that had received cell injections. Inside the fibrotic tissue, trichrome and picrosirius collagen staining disclosed regions with decreased collagen density, in which a rich vascular tree was present. The anterolateral wall also showed larger central vessels that ramified into smaller ones, parallel to the cardiomyocyte bundles (Figure 2B). In the anterolateral wall, the peripheral zone of fibrotic areas merged into the

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cardiomyocyte layer and lack̲ᵤ well-defined limits, unlike the fibrotic areas observed in the posterior wall (Figure 2C). No fibrotic tissue was seen in the central area of the septal wall (Figure 2D).
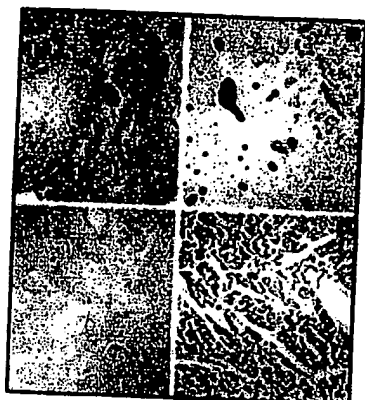

Figure 2. Gomori trichrome stain of anterolateral (A, B), posterior (C), and septal (D) walls. Increased vascular tree is present in B. Original magnification is x40 in A, B, and D; x100 in C.

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Inflammatory cells were rare in the perivascular region: There were occasional isolated small groups of lymphocytes and, very rarely, granulocytes. At the interface between fibrotic tissue and cardiomyocyte bundles, 2 gradients merged: the decreasing blood vessel diameter and the increasing cardiomyocyte size. Very small cardiomyocytes were seen isolated in the fibrotic matrix adjacent to capillaries in the anterolateral wall, together with a progressively increasing number of fibroblastoid cells that were isolated or interspersed in small groups among the cardiomyocyte bundles.

Immunocytochemistry Findings

Immunocytochemical labeling of factor VIII–associated antigen identified a thin endothelial layer of blood vessels in the posterior, septal (Figure 3A), and anterolateral (Figure 3B) ventricular walls. In the anterolateral wall, neither factor VIII nor CD34 was found in the fibroblastoid cell population inside the fibrotic matrix. In the posterior ventricular wall and septum, smooth muscle α-actin was readily identified in blood vessel wall cells. This protein was present both in pericytes and in the smooth muscle cells of the thin vessel wall layer (Figure 3C) in the anterolateral wall. The vascular tree of the anterolateral wall showed intense labeling in the blood vessel walls, which had a marked hypertrophy of smooth muscle cells (Figure 3D). The same staining pattern was present in isolated cells located in the perivascular position and in the adjacent region among cardiomyocytes and fibrotic matrix (Figure 3E). Vimentin was present in the endothelial layer of the anterolateral wall, in the perivascular cells, and in cells adjacent to or in close contact with the cardiomyocytes (Figure 4A). These cells frequently formed an extensive network that permeated the fibrotic matrix and the

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interstitial space among cardiomyocytes (Figure 4B).

Figure 3. Immunocytochemical identification of factor VIII–associated antigen (A, B) and smooth muscle α-actin (C–E) in blood vessel walls of septal (A) and anterolateral (B–E) regions of studied heart, depicting increased vascular density (B) and hyperplasia of perivascular and mural cells (C–E). Ki67 reactivity was rarely present in perivascular cells of anterolateral wall (F). Original magnification x40 in A and B, x400 in C and D, and x1000 in E and F.

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Figure 4. Anterolateral wall that received cell injection therapy. A and B, Immunostaining for vimentin depicted positive reaction in vascular wall and in fibroblastoid interstitial cells. C and D, Immunostaining for desmin showed small groups of intensely reactive cells between blood vessels and cardiomyocytes (C) and small cells inside cardiomyocyte bundles with typical striated cytoskeleton (D). E and F, Immunostaining for troponin showed positive reaction in all mural cells of medium-sized blood vessel. Original magnification is x1000 in A and F; x400 in B–E.

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Desmin was identified in the same cell population. Desmin labeling was less intense in the vascular wall cells and isolated perivascular cells and was more intense in the cells adjacent to cardiomyocytes. On sections perpendicular to the main cardiomyocyte axis, thin desmin-positive fibrils were observed mainly in the submembrane region; on longitudinal sections, a typical transverse banded pattern of desmin was observed (Figure 4C and 4D). Among cardiomyocytes, some

of the small cells had strong, peripheral desmin-stained areas (Figure 4D).

In vascular and perivascular cells in the posterior wall and septum, troponin labeling was negative. In the anterolateral wall, troponin labeling was negative in capillary walls but was positive in the adjacent pericapillary pericytes and in cells migrating into the pericapillary matrix (Figure 4E). In larger vessels, troponin-positive cells were observed in the outer cell layers and adventitia, occasionally forming a continuous troponin-positive cell layer around the vessel (Figure 4F). Isolated cells or small groups of troponin-positive cells were found in the area between the fibrotic tissue and cardiomyocytes and inside the adjacent cardiomyocyte bundles. The intensity of labeling increased in the proximity of cardiomyocytes, where some small fibroblastoid cells disclosed a bright cytoplasm homogeneously labeled for troponin (Figure 5A). Occasionally, such cells had an increased volume, with troponin labeling restricted to the periphery; the central area was filled by a troponin-negative cytoskeleton similar to the desmin-stained areas in small cardiomyocytes. In mature cardiomyocytes, troponin-specific antibody labeled the peripheral filamentous and sarcomeric cytoskeleton.

Figure 5. Anterolateral wall that received cell injection therapy. A, Immunostaining for troponin depicted small cardiomyocyte-like cells with intense reaction in peripheral cell area. B–F, Immunostaining for sarcomeric actinin depicted reactivity in mural cells of blood vessel (B–E) and isolated cells among cardiomyocytes with actinin organization similar to that of sarcomeres (E, F). Original magnification is x400 in A and F; x1000 in B–E.

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Labeling of sarcomeric actinin was similar to that of troponin. However, both pericapillary pericytes and mural blood vessel cells in the anterolateral region were negative for sarcomeric actinin in blood vessels that were deeply embedded in the fibrotic scar matrix and that remained distant from cardiomyocyte bundles. The same cells located in vessels adjacent to or embedded between the cardiomyocyte bundles were positive for sarcomeric actinin, as were the isolated cells or small groups of fibroblastoid cells (Figures 5B and 5C). Some of these cells had increased in size and, in their central region, disclosed sarcomeric actinin that was already organized in the typical banded pattern of sarcomeres (Figures 5D and 5E). In this central region, isolated cells barely larger than pericytes

could be observed; only a few sarcomeres were present, suggesting that those isolated cells had acquired some cardiomyocyte characteristics (Figure 5F).

The Ki67 antibody, which identifies cells actively engaged in replication, reacted only rarely with endothelial cells in the posterior wall. In the anterolateral region, the Ki67 antibody also reacted with pericapillary pericytes and with isolated fibroblastoid cells in the surrounding fibrotic matrix (Figure 3F). The overall cell reactivity with Ki67 antibody was relatively low.

▶ Discussion

Accumulating evidence from both experimental animal studies[4-6] and human trials[7-9] indicates that ABMM cell therapy improves myocardial perfusion in patients with ischemic heart disease. At the same time, clinical stem cell therapy research is focusing more on safety than on efficacy. The present report describes the postmortem study of one patient who underwent transendocardial injection of ABMM cells. Accordingly, the major findings in this report pertain to the procedure's safety: No abnormal or disorganized tissue growth, no abnormal vascular growth, and no enhanced inflammatory reactions were observed. In addition, some intriguing histological and immunohistochemical findings were documented: (1) There was a higher capillary density in the cell-treated area than in nontreated areas of the heart. (2) A proliferation of smooth muscle ▬-actin–positive pericytes and mural cells was noted. (3) The aforementioned cells expressed specific cardiomyocyte proteins.

In the postnatal period, new blood vessels form through either vasculogenesis or angiogenesis, in which proliferation of endothelial cells is followed by remodeling of the extracellular matrix and proliferation of blood wall cells.[13-15] Endothelial cells can result from bone marrow–derived progenitors (postnatal vasculogenesis) or from the migration and proliferation of endothelial cells from existing vessels (angiogenesis).[16] Mural cells such as pericytes and smooth muscle cells can be derived from bone marrow mesenchymal cells (stroma), myofibroblasts, and/or fibroblasts.[17] In the neoangiogenic process, pericytes are derived either from cells of adjacent tissues (mobilized by growth factors produced by endothelial cells) or from proliferation of adventitial and pericapillary pericytes and their distal gliding on the abluminal side of the growing blood vessel's basement membranes.[13] The alternative origination of pericytes from mesenchymal stem cells has been proposed and preliminarily confirmed in experimental models.[18] Pericytes may be essential to achieve a physiological angiogenic process with resultant durable blood vessels. In the present case, when compared with the noninjected regions, the cell-injected wall had marked hyperplasia of pericytes and mural cells. The observed hypertrophic pericytes displayed 2 characteristics: First,

although still located in the vascular wall, they expressed specific myocardial proteins and second, they were found in locations that suggested detachment, having migrated into the adjacent tissue and reached proximal cardiomyocytes that were either isolated or in small cell clumps. Closer to cardiomyocytes, the expression of myocardial proteins was enhanced, yielding brighter immunostaining throughout the whole cytoplasm. The significance of these findings remains to be established. However, within the posterior wall, none of the findings was seen, and small blood vessels could only rarely be found.

Notwithstanding the aforedescribed data, the present report has limitations that severely restrict our ability to make conclusions about the role of ABMM cells in myocardial regeneration. The findings could have occurred by chance. It is impossible to exclude the influence of a natural recovery process as the cause for the difference in vascular density between cell-treated and nontreated areas. Comparisons of capillary density among different sections of wall were based on specimens from a single patient. Moreover, this is an isolated, uncontrolled case involving late events after injection of unlabeled cells; it precluded the use of any imaging technique that could have helped to colocalize and identify the presence of stem cell direct descendants within the vessel wall or myocardium. Therefore, the significant difference in vascular density between cell-treated and nontreated areas cannot be extrapolated to a larger population of similar patients. However, the increased vascular density within the cell-injected anterolateral wall accompanied that wall's improvement in perfusion as assessed by SPECT, whereas all other walls remained unchanged.

▶ Conclusion

At 11-month follow-up evaluation, stem cell therapy was not associated with any adverse histological findings. Morphological and immunohistochemical analysis of the area that underwent ABMM cell implantation suggested that that area had more capillaries than nontreated areas and that ABMM cell therapy was associated with hyperplasia of pericytes, mural cells, and adventitia. Some of these cells had acquired cytoskeletal elements and contractile proteins (desmin, troponin, and sarcomeric actinin).

▶ Acknowledgments

assistance with statistical questions.

▶ Footnotes

*Drs Dohmann and Perin are coprincipal investigators. ◘

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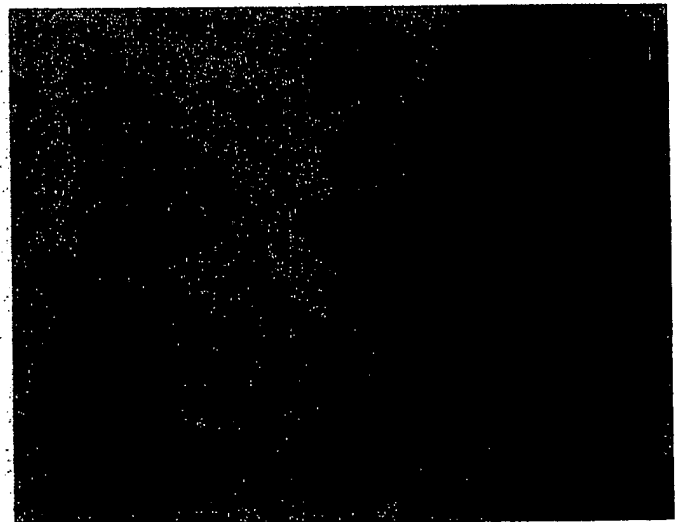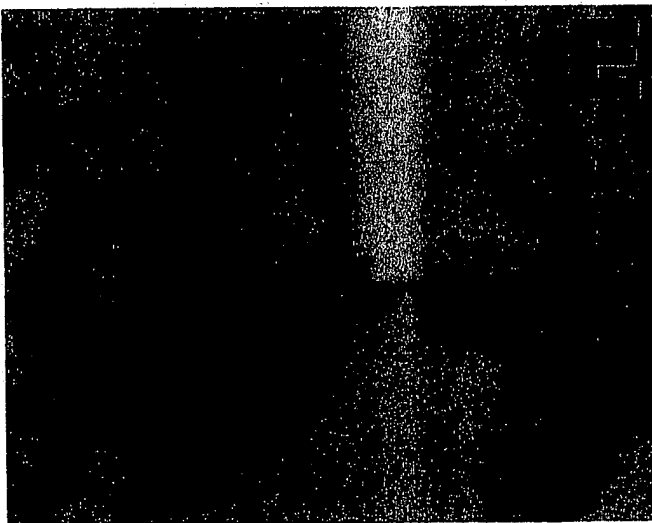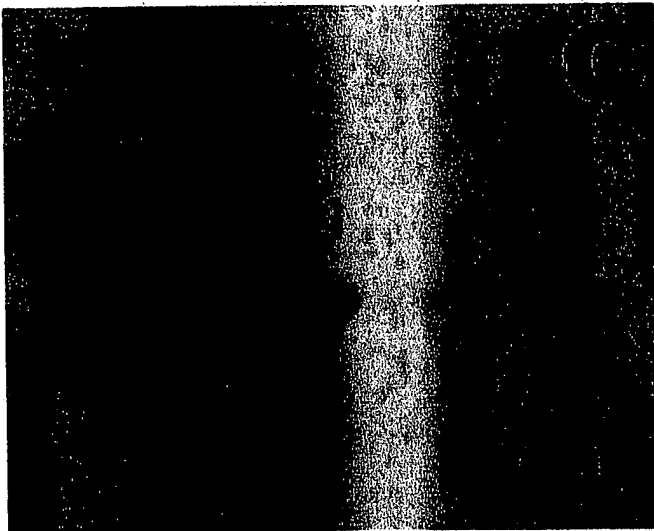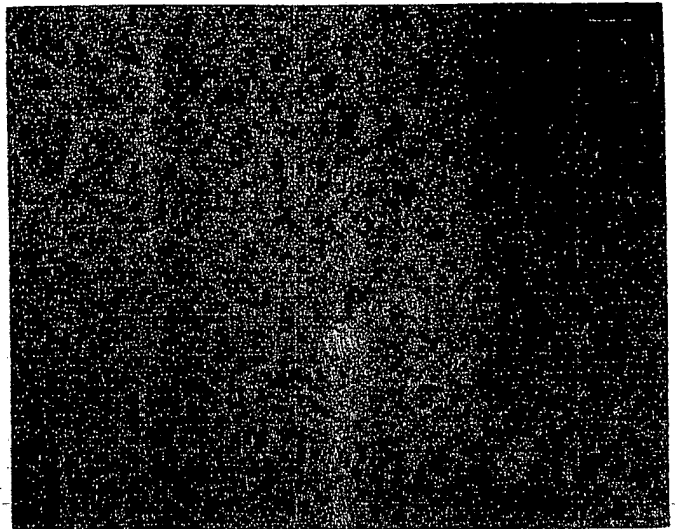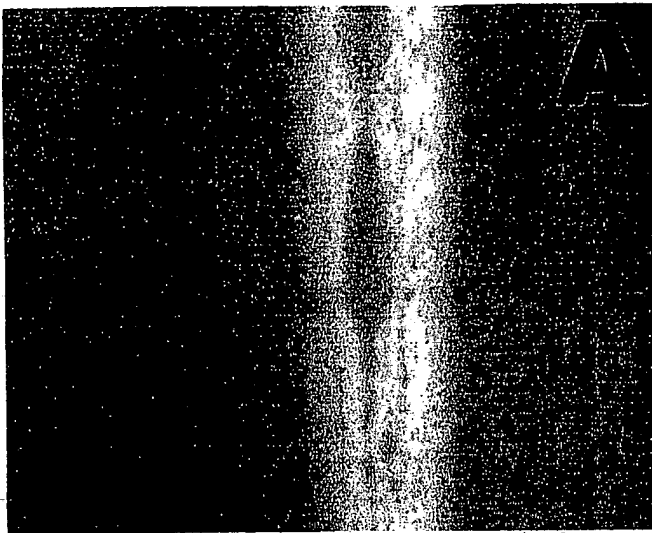**D. J. Prockop and S. D. Olson**
Clinical trials with adult stem/progenitor cells for tissue repair: let's not overlook some essential precautions
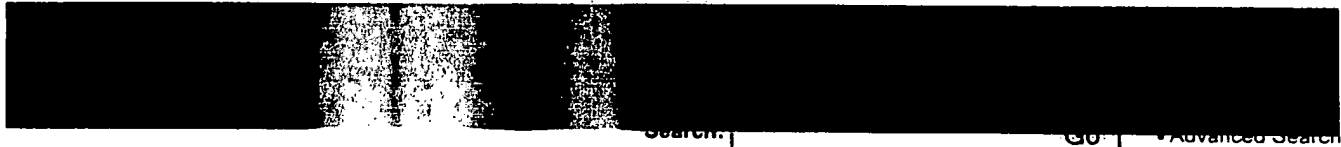Blood, April 15, 2007; 109(8): 3147 - 3151.
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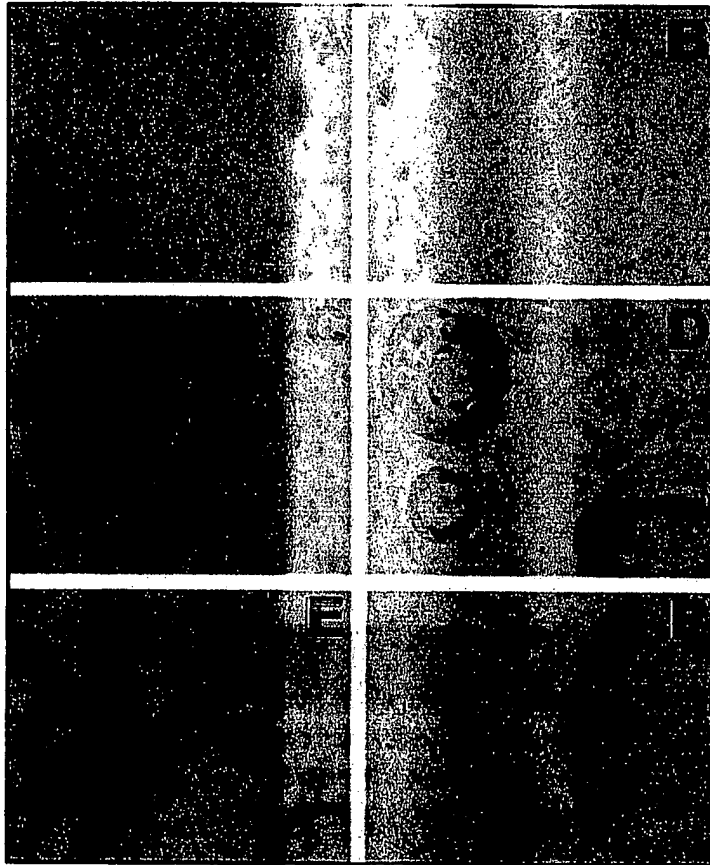
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Figure 3. Immunocytochemical identification of factor VIII–associated antigen (A, B) and smooth muscle α-actin (C–E) in blood vessel walls of septal (A) and anterolateral (B–E) regions of studied heart, depicting increased vascular density (B) and hyperplasia of perivascular and mural cells (C–E).

EVIDENCE APPENDIX

ITEM NO. 30

Strauer et al. 2005 publication in <u>Circulation</u> entitled,
"Regeneration of Human Infarcted Heart Muscle by
Intracoronary Autologous Bone Marrow Cell
Transplantation in Chronic Coronary Artery Disease"cited
by Appellant as Exhibit D in an Amendment
dated November 21, 2005 in co-pending application
Serial No. 09/974,456

(also attached hereto as Exhibit G)

Regeneration of Human Infarcted Heart Muscle by Intracoronary Autologous Bone Marrow Cell Transplantation in Chronic Coronary Artery Disease

The IACT Study

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OBJECTIVES	Stem cell therapy may be useful in chronic myocardial infarction (MI); this is conceivable, but not yet demonstrated in humans.
BACKGROUND	After acute MI, bone marrow-derived cells improve cardiac function.
METHODS	We treated 18 consecutive patients with chronic MI (5 months to 8.5 years old) by the intracoronary transplantation of autologous bone marrow mononuclear cells and compared them with a representative control group without cell therapy.
RESULTS	After three months, in the transplantation group, infarct size was reduced by 30% and global left ventricular ejection fraction (+15%) and infarction wall movement velocity (+57%) increased significantly, whereas in the control group no significant changes were observed in infarct size, left ventricular ejection fraction, or wall movement velocity of infarcted area. Percutaneous transluminal coronary angioplasty alone had no effect on left ventricular function. After bone marrow cell transplantation, there was an improvement of maximum oxygen uptake (VO_{2max}, +11%) and of regional ^{18}F-fluor-desoxy-glucose uptake into infarct tissue (+15%).
CONCLUSIONS	These results demonstrate that functional and metabolic regeneration of infarcted and chronically avital tissue can be realized in humans by bone marrow mononuclear cell transplantation. (J Am Coll Cardiol 2005;46:1651–8) © 2005 by the American College of Cardiology Foundation

Cardiac performance after myocardial infarction (MI) is compromised by ventricular remodeling, which represents a major cause of late infarct-related chronic heart failure and death (1,2). Although conventional drug therapy (e.g., with beta-receptor blockers and/or angiotensin-converting enzyme inhibitors) may delay remodeling, there is no basic

therapeutic regimen available for preventing or even reversing this process. By the use of interventional therapeutics (percutaneous transluminal coronary angioplasty [PTCA], stent), recanalization of the occluded infarct-related artery is possible, thereby improving or normalizing coronary blood flow. However, despite sufficient reperfusion of infarcted tissue, the viability of the infarcted myocardium cannot, or can only insufficiently, be improved in most of these patients (3). Therefore, catheter-based therapy of acute MI is useful for vascular recanalization, but the second and crucial step,

the regeneration of necrotic heart muscle, is not realized by this vascular procedure alone.

Experimental (4) and clinical (5,6) studies have shown recently for the first time that bone marrow mononuclear cells (BMCs) may regenerate damaged myocardium in acute MI in humans. Because the regenerative potential of bone marrow-derived cells ought also to be expected to exist in chronically ischemic heart disease as well (7–12), we have assembled in an ongoing clinical investigation 18 patients with chronic MI to prove this new therapeutic possibility.

METHODS

Study population. All 18 patients (49 ± 11 years) were men and were recruited consecutively from January 2003 until March 2004. They had had transmural MI 27 ± 31 months before, at which point all infarcts had been treated acutely by PTCA and/or stent implantation (Table 1, Fig. 1).

The inclusion criteria were age <70 years, one-vessel disease with an open infarct-related artery at the time of stem cell therapy, sinus rhythm, a clear-cut demarcation of the ventriculographic infarct area, and no coronary bypass surgery. General exclusion criteria were severe comorbidity and alcohol or drug dependency. Although chronically infarcted myocardium usually does not regenerate sponta-

Abbreviations and Acronyms	
BMC	= bone marrow mononuclear cell
CPK	= creatine phosphokinase
ECG	= electrocardiogram
LV	= left ventricular
MI	= myocardial infarction
PET	= positron emission tomography
PTCA	= percutaneous transluminal coronary angioplasty
Tx group	= transplantation group

neously, for comparison a control group, parallel to the recruitment of the stem cell transplantation group (Tx group), was recruited and analyzed, meeting the same inclusion criteria as the stem-cell group. The recruitment of patients was performed according to a randomization procedure in which all patients of the entire chronic infarction group were distributed to the treatment group, where they agreed with all the therapeutic regimen. Alternatively, all patients of the chronic infarction group who refused the therapeutic regimen (bone marrow puncture and aspiration, intracoronary cell transplantation, and another cardiac catheterization) were allocated to the control group. All medications with angiotensin-converting enzyme inhibitors and with beta receptor blockers were maintained constant during the study period.

The cell-treated patients had stable ventricular dynamics for infarct size, ejection fraction, and wall movement velocity of infarcted area at least 9 ± 6 months before cell transplantation. Infarct size at the time of cell therapy showed an amount of 27 ± 8% of the circumference of the left ventricle (LV), determined by ventriculography.

Preparation of BMCs. One day before cell therapy, bone marrow was taken (80 ml from the iliac crest) and mono-

nuclear cells were isolated and identified including CD34-positive cells, AC133-positive cells and CD45/CD14 negative cells (6). The cells were isolated under good manufacturing practice conditions by Ficoll density separation on Lymphocyte Separation Medium (Bio Whittaker, Walkersville, Maryland), before the residual erythrocytes were lysed with H_2O. For overnight cultivation, 1×10^6 BMCs/ml were placed in Teflon bags (Vuelife, Cell Genix, Gaithersburg, Maryland) and cultivated in X-Vivo 15 Medium (Bio Whittaker) supplemented with 2% heat-inactivated autologous plasma. The next day, BMCs were harvested and washed three times with heparinized saline before final resuspension in heparinized saline. Viability was 93 ± 3%. Heparinization and filtration (cell strainer, FALCON) was carried out to prevent cell clotting and microembolization during intracoronary transplantation. These cells were used for therapy. All microbiologic tests of the clinically used cell preparations proved negative. All patients received extensive information about the procedure, which was approved by the ethical committee of our university, and all gave written informed consent.

Administration of BMCs. Following assessment of baseline examinations (coronary angiography, left ventriculography, spiroergometry, ^{99m}Tc-tetrofosmin single-photon emission computed tomography (SPECT) and ^{18}F-fluor-deoxy-glucose (^{18}F-FDG) positron emission tomography (PET), cell transplantation was performed via the intracoronary administration route (6,13) using four to six fractional infusions parallel to balloon inflation over 2 to 4 min of 3 to 5 ml of cell suspension, each containing 15 to 22 $\times 10^6$ mononuclear cells. All cells were infused directly into the infarcted zone through the infarct-related artery via an angioplasty balloon catheter, which was inflated at a low pressure (2 to 4 atm) and was located within

Table 1. Demographic Data of Intracoronary Bone Marrow Stem Cell Transplantation Group and Control Group

Characteristics	Tx Group	Control Group	p
No. of patients	18	18	
Age, yrs	49 ± 11	52 ± 10	NS
Transmural myocardial infarction, months before Tx	27 ± 31	30 ± 34	NS
Coronary angiography			
LAD/LCX/RCA as affected vessel	16/0/2	10/3/5	
No. of patients with stent implantation	16	17	NS
Risk factors			
Diabetes mellitus, %	16	11	NS
Positive family history, %	44	33	NS
Smoker and ex-smoker, %	67	56	NS
Hyperlipoproteinemia, %	89	94	NS
Medication			
Beta-blocker, %	94	89	NS
Angiotensin-converting enzyme inhibitor, %	94	89	NS
Statin, %	94	100	NS
Laboratory parameters			
CPK, U/l	1,504 ± 979	1,489 ± 952	NS
Bone marrow mononuclear cells, n ($10^6\times$)	90		

Values are mean ± SD or number of patients.
CPK = creatine phosphokinase; LAD = left anterior descending coronary artery; LCX = left circumflex coronary artery; RCA = right coronary artery; Tx = intracoronary bone marrow stem cell transplantation.
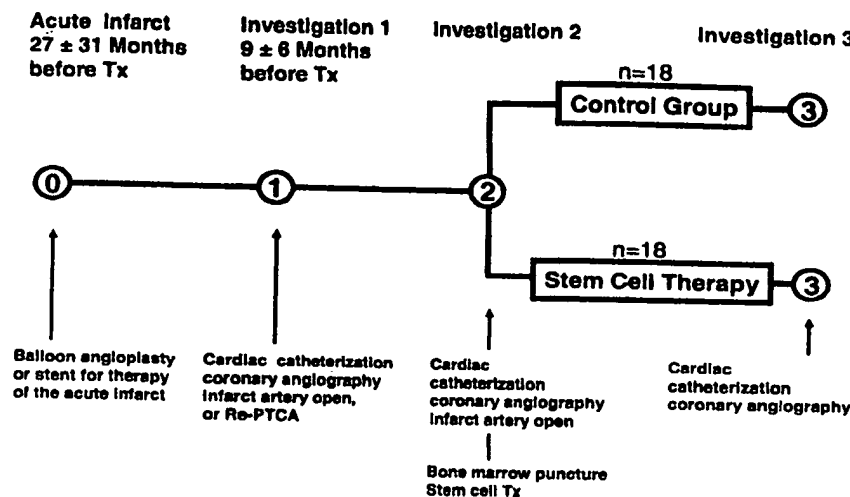
JACC Vol. 46, No. 9, 2005
November 1, 2005:1651–8

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Regeneration of Infarction by BMCs In Chronic CAD

Figure 1. Diagrammatic representation of the algorithm of intracoronary stem cell therapy (Tx) in chronic ischemic heart disease after myocardial infarction. The infarcts occurred 27 ± 31 months before Tx. All infarct patients were treated with percutaneous transluminal coronary angioplasty (PTCA) or with stent implantation. 9 ± 6 months before (investigation 1) coronary angiography (including quantitative left ventriuclography) was performed. If re-stenosis was present, re-PTCA was made. Investigation 2 embraces all patients for the evaluation of coronary morphology after PTCA/stent. Only patients with an open infarct-related artery were included in both groups. Patients who agreed to Tx received within 10 days after investigation 2 bone marrow punctures and Tx by the intracoronary administration route and had altogether five invasive investigations, including two for therapeutic reasons (nos. 0 and 1). Patients who were not eligible for Tx (disagreement with bone marrow puncture and with subsequent Tx) served as a control group. Investigation 3 represents all follow-up measurements 3 months after Tx (Tx patients) or after investigation 2 for control group patients.

the previously stented coronary segments. This prevented backflow of cells and produced stop flow beyond the site of balloon inflation to facilitate high-pressure infiltration of cells into the infarcted zone. Prolonged contact time for cellular migration was also enabled. Three months after catheter-guided cell transplantation, all functional tests were repeated, including coronary angiography and left ventriculography. There were no procedural or cell-induced complications, and there were no side effects in any patient.

Spiroergometry. Aerobic exercise capacity was examined before (<10 days) intracoronary cell transplantation and three months later during follow-up. All patients (n = 18) were subjected to initial bicycle spiroergometry to assess their functional fitness and to determine the limit of safe intensity of exercise. We chose a protocol with an intensified workload up to the symptom-limited maximum (basic load of 50 W, intensification at 25 W, 2-min duration of each workload step). We determined the anaerobic threshold for prescribing a suitable load intensity. During the whole spiroergometry, monitoring by a 12-lead electrocardiogram (ECG) was carried out. The exercise capacity was assessed on the basis of maximum load levels expressed in watts (W_{max}) and maximum peak oxygen uptake (Vo_{2max}).

Coronary angiography and left ventriculography. Coronary angiography and biplane left ventriculography were performed 9 ± 6 months before cell transplantation and also a second time, within 10 days, immediately before cell therapy. The therapeutic follow-up was three months after the treatment. Thus, stable baseline conditions were documented (coronary vessel involvement, ventricular function, and geometry). Cardiac function was evaluated by left ventricular (LV) ejection fraction and by auxotonic myocardial contractility index, evaluated by the wall movement velocity of the infarcted area. The infarct size was calculated according to the method of Sheehan (14) by plotting five axes perpendicular to the long axis of the heart in the main akinetic or dyskinetic segment of the ventricular wall. Systolic and diastolic lengths were then measured by two independent observers, and the mean difference was divided by the systolic duration in seconds.

Quantification of coronary stenosis (restenosis). Cinecoronarangiograms were obtained during stem cell transplantation and at three months thereafter according to standard acquisition guidelines. The angiograms were evaluated by two independent observers and quantitative analysis was performed (15). Standard morphologic criteria were used to characterize the complexity of baseline lesions. The user-defined reference diameter proximal to the stenosis and the minimal luminal diameter within the culprit of the stenosis were used to calculate the percentage of stenosis. A value of 0 mm was assigned for the minimal luminal diameter in case of total occlusion at baseline or follow-up. Restenosis was defined as ≥50% stenosis of the initial target lesion at follow-up. Calculations of restenosis were performed in both groups, with and without stem cell therapy, in the same way, thus enabling evaluation the differential effects of PTCA-guided cell therapy and of PTCA effects alone.

Ventricular function after PTCA in the control group. For the evaluation of a potential effect on the PTCA intervention itself on LV function, all patients in the control group were analyzed with regard to infarct size, ejection fraction, and infarction wall movement velocity.

Table 2. Single Values of Intracoronary Bone Marrow Stem Cell Transplantation Group

Patient Number	Area of Infarction, %			LV Ejection Fraction, %			Infarction Wall Movement Velocity, cm/s*		
	Investigation 1 9 ± 6 Mo Before Tx	Investigation 2 <10 Days Before Tx	Investigation 3 3 Mo After Tx	Investigation 1 9 ± 6 Mo Before Tx	Investigation 2 <10 Days Before Tx	Investigation 3 3 Mo After Tx	Investigation 1 9 ± 6 Mo Before Tx	Investigation 2 <10 Days Before Tx	Investigation 3 3 Mo After Tx
1	26	26	22	56	55	60	0.88	0.77	0.82
2	28	29	26	45	43	49	2.06	1.88	2.13
3	16	16	5	64	65	71	1.45	1.50	2.10
4	27	25	14	48	50	65	1.20	1.25	2.88
5	16	14	11	66	69	71	2.25	2.77	3.75
6	16	13	6	64	66	73	1.50	1.77	2.55
7	15	18	11	57	55	63	2.78	2.65	3.13
8	28	28	20	43	44	49	3.15	3.25	4.25
9	27	27	11	46	46	64	1.61	1.65	3.30
10	20	17	14	56	58	62	2.21	2.45	3.13
11	28	25	17	42	38	52	1.91	1.88	3.00
12	33	28	21	44	47	54	2.28	2.62	3.50
13	39	37	27	50	51	59	1.25	2.50	4.90
14	29	33	27	62	62	61	1.20	1.33	2.70
15	37	37	31	48	43	53	1.83	1.56	2.50
16	29	29	24	53	54	58	1.25	1.06	3.06
17		41	35		48	55		1.66	3.00
18		35	25		45	53		0.94	1.94
Mean	26	27	19	53	52	60	1.80	1.86	2.92
SD	7	8	9	8	9	7	0.63	0.70	0.91

*Calculated from left ventriculography.

LV = left ventricular; Mo = Months; other abbreviations as in Table 1.

Nuclear cardiologic investigations (PET and SPECT). ^{18}F-FDG-positron emission tomography (^{18}F-FDG PET) was performed with a Scanditronix SCX 4096 WB-Scanner (FWHM = 6 mm transaxial, axial field of view = 4.6 cm). Patients received an oral glucose load of 1 g/kg body weight 80 ± 30 min before the intravenous application of ^{18}F-FDG (380 ± 60 MBq). The ^{18}F-FDG was administered at the time of decrease of blood glucose level <130 mg/dl. An initial transmission scan was obtained using a ^{68}Ga-filled pin source to correct the subsequent emission scans for attenuation. The data acquisition was started 45 min after administration of FDG. Image data were recorded with a 256 × 256 matrix in 3 consecutive bed positions over 15 min per position. The data were reconstructed back-projected with a Hanning filter (5 mm).

^{99m}Tc-tetrofosmin SPECT. Sixty minutes after intravenous injection of 600 ± 140 MBq of the perfusion-marker ^{99m}Tc-tetrofosmin under a "rest" condition, the images were obtained using a SPECT scanner with double-head detector (PRISM 2000, Marconi/Phillips), a low-energy, high-resolution collimator, and a 128 × 128 matrix. Image data were collected over 360° at 3° every 30 s. The images were reconstructed backprojected with a low-pass filter (order 12, cutoff 0.2).

PET and SPECT evaluation. Normalized values for FDG uptake and perfusion were calculated by comparing regional with maximum tracer uptake on the reconstructed images. We performed a regional analysis of glucose metabolism and perfusion using a set of standardized, individually adjusted circular regions of interest (diameter 18.06 mm, surface 256 mm^2). The reconstructed metabolic and perfusion images were realigned for each patient (MPI-Tool, version 3.0; Advanced Tomo Vision, Erftstadt, Germany) and were resliced according to cardiac axis (short-axis and horizontal and vertical long-axis views). The regions were positioned immediately neighboring, with no overlap, according to an overlay of the co-registrated metabolic and perfusion images. The regions covered the infarct lesion as well as normal myocardium. In this way, we generated templates of regions for each patient, which could be used for the evaluation of metabolism and perfusion, before and after BMC transplantation without further modification. According to Segall et al. (16), regions with a normalized FDG uptake <50% were rated as transmural scar and regions with an uptake of 50% to 60% as non-transmural scar.

Further analysis was restricted to regions with FDG uptake <60% in the PET scans, pursuant to our intention to focus on the effects of BMC transplantation on scar tissue.

Safety parameters. To assess any inflammatory response and myocardial reaction after cell therapy, white blood cell count, the serum levels of C-reactive protein (CRP) and of creatine phosphokinase (CPK) were determined immediately before as well as after treatment. Additional analysis was done directly after transplantation and three months later: ECG at rest, 24-h Holter ECG, and echocardiography.

Statistical analysis. All data are presented as mean ± SD. Statistical significance was accepted when p < 0.05. Intra-individual comparison of variables of investigation 1 (9 ± 6 months before cell transplantation for Tx group, 9 ± 5 months before investigation 2 for control patients) and investigation 2 (<10 days before cell transplantation for Tx group, no transplantation for control patients) and of variables of investigation 2 and follow-up investigation 3 (3 months after cell therapy for Tx group, 8 ± 5 months after investigation 2 for control patients) was performed using Wilcoxon rank-sum test. The missing values (Table 2) were omitted and not calculated for statistical analysis. The p values (by analysis of variance) have been given for LV ejection fraction, area of infarction, and infarction wall movement velocity. Statistical analysis was performed with SPSS-Windows 10.1 software.

RESULTS

Three months after intracoronary cell therapy, the infarct size was reduced by 30%, whereas the global LV ejection fraction increased by 15% and regional infarct wall movement velocity by 57% (Tables 2 and 3). In parallel, the clinical performance improved (Table 4), as evidenced by a higher work load demonstrated by a 11% increase in maximum oxygen uptake (Vo_{2max}). SPECT investigation presented enhanced tetrofosmin uptake in the infarcted zone by 5%, and PET examination showed enhanced glucose uptake in the infarcted zone by 15%, demonstrating regeneration of formerly avital, chronically infarcted heart muscle (Fig. 2). An unchanged or even impaired LV function was not observed in any patient.

In the control group (18 patients with chronic MI, but without stem cell therapy) no significant changes were observed in infarct size, LV ejection fraction, or wall

Table 3. Cardiac Parameters in the Transplantation Group and in Control Group at the Three Investigation Time Points

	Area of Infarction, %			LV Ejection Fraction, %			Infarction Wall Movement Velocity, cm/s		
	Control Group	Tx Group	p Value*	Control Group	Tx Group	p Value*	Control Group	Tx Group	p Value*
Investigation 1	25 ± 9	26 ± 7	0.99	53 ± 10	53 ± 8	0.87	1.95 ± 0.66	1.80 ± 0.63	0.57
Investigation 2	27 ± 9	27 ± 8	0.83	51 ± 10	52 ± 9	1.00	1.88 ± 0.76	1.86 ± 0.70	0.94
Investigation 3	26 ± 9	19 ± 9	0.02	52 ± 10	60 ± 7	0.02	1.91 ± 0.79	2.92 ± 0.91	0.001

*Analysis of variance.
Abbreviations as in Table 1.

Table 4. Positron Emission Tomography and Spiroergometry Before and After Stem Cell Therapy in Chronically Infarcted Myocardium

	^{18}F-FDG-Positron Emission Tomography		VO_{2max}, Spiroergometry	
	FDG Uptake, %	Difference in %	ml/min	Difference in %
Investigation 1	none		none	
Investigation 2	43.8 ± 8.0	+ 15	1,602 ± 533	+ 11
Investigation 3	50.5 ± 11.6		1,776 ± 523	
p (Wilcoxon test)	0.012		0.0001	

^{18}F-FDG = ^{18}F-fluor-deoxy-glucose; VO_{2max} = maximum oxygen uptake.

movement velocity of the infarcted area (Figs. 3A to 3C). Electrocardiogram at rest and on exercise and 24-h Holter ECG revealed no rhythm disturbances at any time point. Only 1 patient (from 18 cell-treated patients, 6%) developed relevant restenosis due to quantitative angiographic criteria. The restenosis could be treated adequately by stent implantation. The other 17 patients showed good patency rates without restenosis after PCI and cell transplantation. They also revealed no alterations in LV function 8 ± 5 months after PTCA.

There was no inflammatory response or myocardial reaction (white blood cell count, CRP, CPK) after cell therapy, despite a moderate increase in CRP (before cell transplantation 0.58 ± 0.48 mg/dl, after cell transplantation 1.07 ± 0.73 U/l, p = 0.002), which is usual after bone marrow puncture and/or cardiac catheterization.

DISCUSSION

The results of these investigations demonstrate, for the first time, that the intracoronary transplantation of autologous bone marrow mononuclear cells may reduce infarct size and improve LV function as well as myocardial glucose uptake in chronic ischemic heart disease attributable to chronic MI (5 months to 8.5 years old). Infarct size decreased in all patients and cardiac performance (ejection fraction, wall movement velocity of infarcted area, maximum oxygen uptake, and exercise tolerance) and myocardial metabolism (FDG-PET) improved, all being between 11% and 57%. Furthermore, it is noteworthy that there were no complications immediately or three months after cell transplantation, especially that there was no cardiac arrhythmia and no signs of cardiac or systemic inflammation were present.

The effects of stem cell transplantation on infarct size, cardiac function, and contractility demonstrate significant improvement of these three parameters in the therapy group (before and after stem cell therapy) as well as in the comparison between the stem cell therapy group and the control group, thus giving evidence for a beneficial therapeutic effect of stem cell therapy on cardiac performance in chronic MI.

Patients in both the stem-cell group and the control group were recruited in parallel to each other and consecutively between January 2003 and March 2004. They all (n = 36) fulfilled the same inclusion criteria. Thus, representative patient characteristics were present for the stem cell group (n = 18) and the control group (n = 18) as well as in comparing both of them. Moreover, two subsequent investigations before stem cell transplantation have been performed for each patient: investigation 1 and 2 demonstrated the stability of LV dynamics before cell therapy (9 months respectively 10 days before transplantation) and investigation 3 compared the effects of stem cell therapy with the control group. The stable hemodynamics during the preceding 9 ± 6 months before stem-cell therapy and the stable hemodynamics within the control group at all three points of investigation underline the significant alterations of the left ventriculography-derived parameters investigated after stem cell transpantation.

The regenerative potential of bone-marrow–derived stem cells may be explained by any of four mechanisms: 1) direct cell differentiation from mononuclear cells to cardiac myocytes (17), 2) cytokine-induced growing and increase of residual viable myocytes, especially within the border zone of the infarcted area (18), 3) stimulation of intrinsic myocardial stem cells (endogenous stem cells) (19,20), and 4)
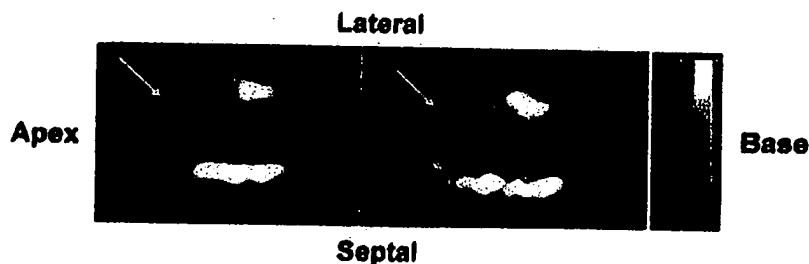
Lateral

Septal

Figure 2. Representative illustration of ^{18}F-FDG-positron emission tomography (PET) before (above) and 3 months after (below) cell therapy in the transversal (left) and longitudinal (right projection) in a 30-year-old male patient with an 8-month-old anteroapical infarction. Note the restoration of glucose uptake (below) within the infarcted area of the formerly completely avital anteroapical myocardium.
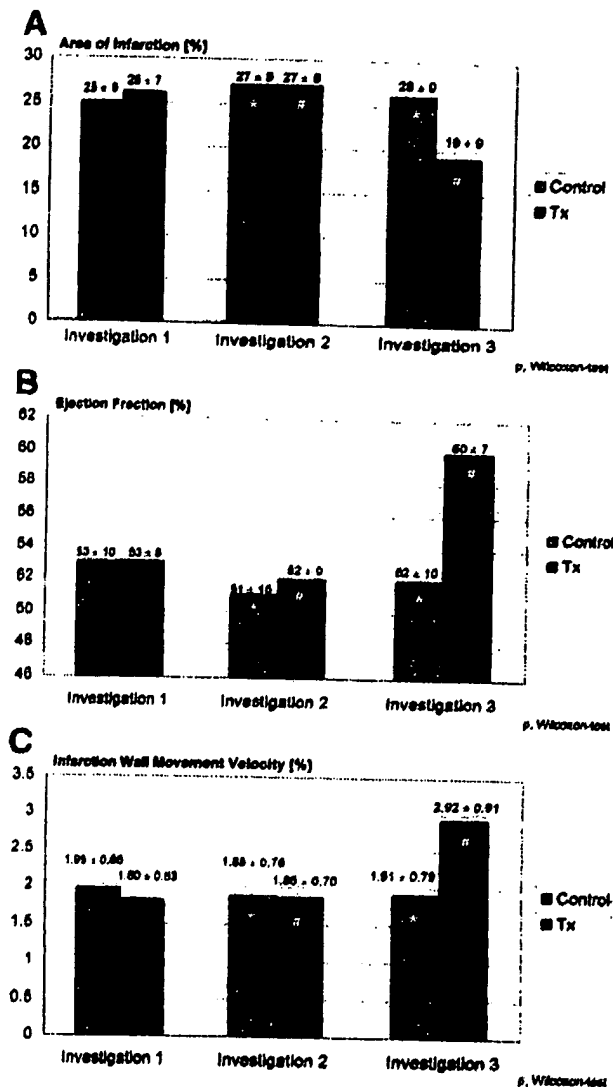
A

B

C

Figure 3. Illustration of the mean values of (A) area of infarction, (B) ejection fraction, and (C) infarction wall movement velocity, determined by quantitative left ventriculography in both groups (control group vs. transplantation [Tx] group) at the point of time: investigations 1, 2, and 3. Comparision of both groups with chronically infarcted myocardium (conrol group vs. Tx group), n = 18 patients. Investigation 1 was 9 ± 6 months before cell transplantation (controls: 9 ± 5 months before percutaneous transluminal coronary angioplasty [PTCA]); investigation 2 within 10 days before cell tranplantation (controls: at the time point of PTCA) and investigation 3 was three months after cell transplantation (controls: 8 ± 5 months after PTCA). Note the significant decrease of infarct size and the increase in ejection fraction and in contractility (infarction wall movement velocity) 3 months after cell therapy in comparison with the control group. *p = not significant (investigation 2 vs. investigation 3); #p = 0.001 (investigation 2 vs. investigation 3).

induction of cell fusion between transplanted bone marrow cells and resident myocytes (21–24).

Transdifferentiation has been described by previous investigators (4); however, it has been questioned by recent experimental studies (25). The influence of cytokines has

shown to restore coronary blood vessels and muscle cells after experimental myocardial infarction. This regeneration of blood vessels and muscle cells is most pronounced in the border zone of ischemic and/or infarcted tissue (26), demonstrating an enhancement of mitotic cells and cell cycles up four-fold, when compared to areas remote from the necrotic myocardium. Moreover, mononuclear bone marrow stem cells contain a lot of cytokines (VEGF, insulin-like growth factor, platelet-derived growth factor, and so on), thereby stimulating residual normal myocytes for regeneration and proliferation and instrinsic myocardial stem cells (endogenous stem cells) for cell regeneration and for cell fusion (27–31).

Mitotic indexes are three to four times more frequent within the border zone of myocardial necrosis when compared with non-injured heart muscle (26). Moreover, 20% to 40% of intracoronarily transplanted bone-marrow–derived stem cells may be accumulated within the border zone of MI. There were no signs of apparent microcirculation disturbances because all patients had Thrombolysis In Myocardial Infarction flow grade 3. Thus, it is conceivable that in MI the border zone represents the optimum "niche" for exogenously transplanted stem cells, stimulating mitosis rates and heart muscle regeneration, preferably originating in and expanding from these areas. Cell fusion may also contribute to heart muscle regeneration, which takes its origin from the border zone, expanding gradually to the necrotic core of the infarcted area.

Our study cannot determine which cell-biologic and molecular mechanims are responsible for heart muscle repair or which of the studied factors may play the predominant role. However, the final functional outcome of this cell therapy demonstrates three main target effects: improvement in muscle function (pumping ability and contractility), myocardial perfusion (SPECT), and myocardial glucose metabolism (PET), thus giving evidence that heart muscle repair must have taken place by this intracoronary bone marrow cell transplantation procedure.

The clinical significance of this novel therapeutic approach may embrace a large number of patients with chronic coronary artery disease, preferably after previous or longstanding MI. It is conceivable that remodeling after infarction may be ameliorated or even stopped by this procedure. Thus, cell therapy may represent a new option of basic and causal therapy in chronic infarcted myocardium. It is an open question whether variations of the amount and kind of bone marrow cells, the administration technique, and the transplantation procedure itself, by enhanced environment and improvement of the angiogenic micromilieu, can further improve the milieu-dependent differentiation or regeneration of bone marrow cells in chronic infarcted heart disease. Therefore, our clinical results represent a stable basis to proceed to the next necessary step: to a larger prospective randomized study.

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EVIDENCE APPENDIX

ITEM NO. 31

Final Office Action dated September 22, 2006,
page 22, first paragraph, issued in co-pending application
Serial No. 09/836,750

(also attached hereto as Exhibit H)

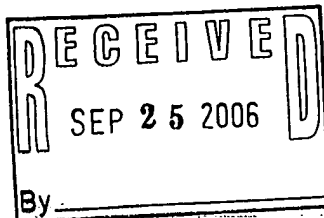| APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/836,750	04/17/2001	James P. Elia	1000-10-C01	7239

7590 09/22/2006	EXAMINER	
Gerald K. White	KEMMERER, ELIZABETH	
GERALD K. WHITE & ASSOCIATES, P.C.		
205 W. Randolph Street, Suite 835	ART UNIT	PAPER NUMBER
Chicago, IL 60606	1646	

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DATE MAILED: 09/22/2006

Please find below and/or attached an Office communication concerning this application or proceeding.

PTO-90C (Rev. 10/03)

	Application No.	Applicant(s)
Office Action Summary	09/836,750	ELIA, JAMES P.
	Examiner	Art Unit	
	Elizabeth C. Kemmerer, Ph.D.	1646	

-- *The MAILING DATE of this communication appears on the cover sheet with the correspondence address* --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE _3_ MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
  Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1) ☒ Responsive to communication(s) filed on _26 June 2006_.

2a) ☒ This action is **FINAL**.   2b) ☐ This action is non-final.

3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4) ☒ Claim(s) _6-236,238-253 and 256-287_ is/are pending in the application.

    4a) Of the above claim(s) _6-235 and 240-242_ is/are withdrawn from consideration.

5) ☐ Claim(s) _____ is/are allowed.

6) ☒ Claim(s) _236,238,239,243-253 and 257-287_ is/are rejected.

7) ☐ Claim(s) _____ is/are objected to.

8) ☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9) ☐ The specification is objected to by the Examiner.

10) ☐ The drawing(s) filed on _____ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.

    Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

    Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11) ☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

    a) ☐ All   b) ☐ Some * c) ☐ None of:

      1. ☐ Certified copies of the priority documents have been received.

      2. ☐ Certified copies of the priority documents have been received in Application No. _____.

      3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

    * See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)

2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)

3) ☐ Information Disclosure Statement(s) (PTO/SB/08)
    Paper No(s)/Mail Date _____.

4) ☐ Interview Summary (PTO-413)
    Paper No(s)/Mail Date. _____ .

5) ☐ Notice of Informal Patent Application

6) ☐ Other: _____.

DETAILED ACTION

Status of Application, Amendments, And/Or Claims

The amendment received 26 June 2006 has been entered in full. Claims 1-5, 237, and 254-256 are canceled. Claims 6-235 and 240-242 remain withdrawn from consideration as being directed to a non-elected invention. Claims 236, 238, 239, 243-253, and 257-287 are under examination.

The fourth supplemental declaration of Dr. Heuser under 37 CFR 1.132 and third supplemental declaration of Dr. Lorincz under 37 CFR 1.132 submitted with the response have been entered. A copy of the third supplemental declaration of Dr. Heuser under 37 CFR 1.132 has also been received.

The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

As an initial matter, it is noted that Applicant comments upon alleged procedural errors. The record has been reviewed and no errors in procedure have been noted. Therefore, these comments will no longer be addressed further.

35 U.S.C. § 112, First Paragraph, New Matter

Claims 248, 249, 252, and 274-279 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. This is a new matter

second piece of evidence, Merck has to do with cancer and does not appear to be relevant. The third piece of evidence, NIH report, lists results of a web search for "nonspecific growth factor" and also appears to be irrelevant to the issue at hand. The last piece of evidence, Exhibit III in the after final amendment, reviews traditional use of cells for cancers and immunotherapy, and newer uses as gene therapy vehicles. None of these treatments involve the systemic administration of cells to repair a distant organ.

Applicant argues that administration of cells is old in the art. This point is conceded.

Applicant argues that the examiner's statement regarding Deb et al., wherein it was acknowledged that cells administered intravenously could migrate to the art, should end all speculation regarding enablement of the claimed invention. This has been fully considered but is not found to be persuasive because Deb et al. do not demonstrate that cells can migrate to the heart in sufficient quantities to repair any defects. Deb discloses that only $0.23 \pm 0.06\%$ of the cardiomyocytes were from the transplanted cells. Such numbers of cells are greatly insufficient to achieve the effects required by the claims. As evidence of this, Strauer 2002 administered 6 to 7 fractional high-pressure infusions of 2 to 3 mL cell suspension, each of which contained 1.5 to 4 X 10^6 mononuclear cells directly to the infarct site in order to achieve their effects. In fact, Strauer 2002 specifically points to shortcomings of intravenous administration at p. 1917. The evidence as a whole indicates that intravenous administration of cells to repair a dead or damaged portion of a heart has not yet been achieved due to the obstacles involved with getting sufficient numbers of cells to the dead/damaged site and